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Toxicity of Tunicamycin to Cultured Brain Neurons: Ultrastructure of the Degenerating Neurons

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Abstract Our previous study has shown that tunicamycin irreversibly downregulates the expression of $GABA_AR$ and causes cell death in cultured brain neurons by biochemical and light microscopic methods. In this study, we examined mechanisms underlying the degeneration of the neurons mainly employing electron microscopic analysis. Cultured neurons derived from embryonic chicken brains were incubated with 5 μg/ml of tunicamycin (TM) for 24 h, followed by continual incubation or removal of TM for additional 3 h or 24 h. Neurons treated with TM for 24 h showed dilated rough endoplasmic reticulum (rER), nuclear envelope and components of Golgi apparatus, in addition to the degranulation of rER and disaggregation of ribosomal rosettes. In neurons subjected to the prolonged incubation, some ribosomes reattached to the membranes of rER; the polyribosomes reappeared, and the swelling of Golgi apparatus subsided. However, the distention of rER persisted, and an uncommon spindle-like structure appeared in the perikarya. This structure is implicated to involve the neuronal degeneration. Moreover, extracellular cell debris was increased with time of incubation. The ratio of the light neurons, defined as containing lower cytoplasmic matrix density than the untreated control, decreased from 28% at 3 h to 3% at 24 h after the removal of TM, and 45% at further 3 h to 6% at further 24 h incubation of TM, whereas dense neurons only appeared in the two 24 h groups, as 44% and 34%. The light neurons resemble necrotic cells, but the dense neurons exhibit distinct morphological features from necrosis and apoptosis. The gel electrophoresis assay revealed the absence of DNA fragmentation in all cultures. In addition, whole cell recordings exhibited a 40% decrease of the GABA-elicited current in the neurons exposed to TM for 24 h. The results indicate irreversible toxicity of chronic TM treatment to the neurons and suggest differential mechanisms for the neuronal death among various populations of cells. It is evident that the N-glycosylation plays a critical role for neuronal survival. J. Cell. Biochem. 74:638–647, 1999.

Key words: electron microscopy; rough endoplasmic reticulum; spindle-like structure; DNA gel electrophoresis; $GABA_A$ receptor; whole-cell recording; neuronal death

The administration of tunicamycin (TM), an antibiotic, has been shown to generate fatal neurological diseases in animals [Vogel et al., 1982; Jago et al., 1983]. The phenomena of the pyknosis of nuclei and cytoplasmic vacuolation were considered to represent necrosis of Purkinje cells and other types of brain cells in the TM-injected animals [Finnie and O'Shea, 1988; Leaver et al., 1988]. TM treatment likewise resulted in the diminished integrity of cell membrane and cytoplasmic organelles, similar to the cytolysis of necrosis, in cultured brain neurons of mouse embryos [Lodin et al., 1984]. In contrast, recent studies have proposed that TM kills cultured sympathetic neurons and cerebellar granular cells by inducing programmed cell death, or "apoptosis," according to the nuclear condensation observed under a light microscope by bisbenzimide staining and the protection by cycloheximide from the cell death [Chang and Korolev, 1996; Chang et al., 1997]. Nonetheless, ultrastructural features and DNA fragmentation for apoptosis remain unknown in these reports.

On the other hand, TM has been widely used as a research tool for analyzing cellular events involving protein glycosylation, because it pre-

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vents the N-linked glycosylation. We have previously employed TM to study the significance of N-glycosylation to the expression of the γ-aminobutyric acid$_A$ receptor ($GABA_AR$), the major inhibitory neurotransmitter receptor in vertebrate brain [Lin et al., 1998]. The receptor is a multi-subunit membrane glycoprotein and modulates neuronal excitability through its integral Cl^- ion channel by binding with GABA [Rabow et al., 1995]. We have found that 24 h incubation of 5 μg/ml of TM downregulates the number of binding sites of ^{3}H-flunitrazepam or ^{3}H-muscimol on $GABA_AR$ and changes the subcellular distribution of the receptors in cultured brain neurons [Lin et al., 1998]. Under this TM treatment, the cells remained viable as revealed by Trypan blue exclusion experiment. However, significant cell death started at 3 h after TM was removed, because the ratio of cells ingested the dye increased and the level of the radioligand binding decreased. Mechanisms underlying the neuronal death await further investigation.

Neuronal death was also induced in culture by other agents, including the agonists for glutamate receptors (GluRs). The application of glutamate, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), or kainate produced degenerating neurons exhibiting necrotic features of swollen cell body, rough endoplasmic reticulum (rER), and mitochondria, and disrupted membranes [Regan et al., 1995; Gwag et al., 1997]. Furthermore, direct injection of NMDA into the adult rat striatum generated neurons with apoptotic characteristics, the nuclear margination of compacted chromatin and cytoplasmic condensation in the retrosplenial cortex and mammillary nucleus, whereas necrotic pyramidal cells were seen in the hippocampus [Van Lookeren Campagne et al., 1995]. However, atypical morphology of apoptosis and necrosis occurred for neurons in the neocortex, dentate gyrus, and other areas in newborn rats after the injection of kainate or AMPA [Portera-Cailliau et al., 1997]. Whether TM induces such a variety of mechanisms for neuronal death is unclear.

It has been shown previously that effects of TM treatment involve electrophysiological changes of ion channels. The amplitude of GABA-elicited currents was reduced by chronic incubation with TM in *Xenopus* oocytes injected with the rat brain mRNAs [Sumikawa et al., 1988]. GluR agonist-induced current was also blocked by the agent in the same preparation [Mußhoff et al., 1992]. Nevertheless, the evidence for electrophysiological alterations is lacking in cultured neurons treated with TM.

Thus, as a continuation of our earlier study, the goal of the present study is to further investigate mechanisms for the death of cultured brain neurons induced by TM. Ultrastructural and biochemical analyses were carried out by utilizing electron microscopy and DNA gel electrophoresis. In addition, the effect of TM on the functional expression of $GABA_AR$ was examined by recording GABA-elicited whole-cell currents of the neurons.

MATERIALS AND METHODS

Neuron Culture and Drug Treatment

Neuron cultures were prepared from brains of 7-day chicken embryos as described previously [Roca et al., 1990; Yin and Yang, 1992]. Briefly, dissociated neurons were plated on collagen-coated culture dishes and maintained in culture for 7 days. Cytosine arabinoside (1 μM; Sigma, St. Louis, MO) was used to prevent excessive proliferation of non-neuronal cells. The cultures were incubated with 5 μg/ml of TM (MW, 800; Sigma) for 24 h. Thereafter, a number of cultures were fed with fresh medium with or without 5 μg/ml of TM and incubated for additional 3 or 24 h.

Electrophysiology

The electrophysiological properties of the neurons were examined by the whole-cell patch-clamp recording described before [Hamill et al., 1981]. Prior to recording, the neurons were washed with the external recording medium, 142 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, and 1.8 mM $CaCl_2$ in 10 mM HEPES, pH 7.4. The recording electrode with a resistance of 5 MΩ was filled with 145 mM CsCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 5 mM EGTA in 5 mM HEPES, pH 7.2. In all recordings, neurons were voltage-clamped at -60 mV. Whole-cell currents elicited by topical application of 100 μM of GABA (Sigma) were recorded by a patch-clamp amplifier (Dagan 8900) and stored on a videotape for later playback onto a storage oscilloscope (Tektronix 5113) or an oscillographic recorder (Gould RS3200). To verify that GABA-elicited currents are mediated by $GABA_AR$, a number of cultures received bath application of 20 μM bicuculline (RBI, Natick, MA), the $GABA_AR$ antagonist, at

10 min before the onset of the GABA application. A Data 6100 waveform analyzer (Data Precision) was used to analyze the amplitude of the currents. Data are presented as means ± standard errors. Significance in difference among the data was analyzed using Student's-*t*-test.

Transmission Electron Microscopy

Cells grown on 35-mm culture dishes were fixed with 2.5% glutaraldehyde in PBS for 30 min at room temperature, washed twice with PBS, and fixed again with 1% OsO_4 for 10 min. The cells were then dehydrated in a graded series of ethanol and embedded in Epon. Ultrathin sections of about 70 nm thickness were obtained, stained with 1% uranyl acetate and 1% lead citrate, and examined under a Jeol JEM-2000EXII electron microscope. For quantification of the results, two dishes were used for each control or respective conditions of TM treatment (n = 3/group); neurons were selected from five evenly distributed 1 mm^2 areas in each dish. The neurons were visually classified into three categories, according to the intensity of electron density of the cytoplasmic matrix. The level of the cytoplasmic density was defined as "intermediate" in control neurons. "Light neurons" referred to neurons with lower level of the cytoplasmic density than that of the untreated control, whereas "dense neurons" possessed increased density of the cytoplasmic matrix. Corresponding levels in the density of nucleoplasmic matrix were also seen for the three divisions of neurons.

DNA Gel Electrophoresis

DNAs were isolated from cultured neurons following a previous method with modifications [Blin and Stafford, 1976]. Cells plated on 100-mm culture dishes were washed with 0.8% NaCl, 0.02% KCl, and 0.0015% phenol red in 0.3% Tris base, pH 7.4. The cells on each dish were then incubated with 10 ml of lysis buffer, containing 20 µg/ml RNase A (Sigma), 0.5% SDS, and 0.1 M EDTA in 10 mM Tris-HCl (pH 8.0), at 37°C for 1 h. Proteinase K (Sigma) was then added to the cell lysate to a final concentration of 100 µg/ml, followed by incubation of the sample at 55°C overnight. After mixing with an equal volume of phenol/chloroform (1:1; v/v), the sample was centrifuged at 2,000*g* for 5 min. The aqueous supernatant was mixed again with chloroform before centrifugation at 2,000*g* for 5 min. Subsequently, the DNA in the second aqueous supernatant was precipitated with isopropanol containing 0.3 M sodium acetate, and collected by centrifugation at 20,000*g* for 20 min. The pellet was washed with 70% ethanol, air dried, and dissolved with 0.1 M EDTA in 10 mM Tris-HCl, pH 8.0. The samples were mixed with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 15% Ficoll-400), and subjected to electrophoresis on 2% agarose gels containing 0.5 µg/ml of ethidium bromide. A 100-bp ladder was used as the molecular weight standard. After electrophoresis, the illuminated gels were photographed with a Polaroid camera on a UV transilluminator.

RESULTS

Electrophysiological Observations

In response to topical GABA application, patch-clamp recordings showed an average current amplitude of 2.5 ± 0.3 nA (n = 18) in untreated control neurons. This GABA-evoked current was blocked by 90 ± 0.1% (n = 8) by bath application of 20 µM bicuculline, the $GABA_AR$ antagonist, at 10 min before the onset of GABA application. Therefore, it is demonstrated that the current is primarily mediated by the receptor. Exposure of the neurons to 5 µg/ml of TM for 24 h reduced the level of the GABA-elicited current to 1.5 ± 0.2 nA (n = 18), about 60% of the control value (Fig. 1).

Morphology of Cultured Neurons Treated With TM

Electron micrographs revealed that neurons in the 7-day control culture contained predominantly large nuclei with prominent nucleoli. Golgi apparatus was mostly composed of flattened stacks of cisterns and vesicles. Conspicuous ribosomes usually disseminated in the cytoplasm as rosette structures and appended on the membranes of rER and the outer membrane of nuclear envelope (Fig. 2A–C).

Incubation of the neurons with 5 µg/ml of TM for 24 h induced profound indentations of the nuclei, an increase in the content of condensed chromatin, and the dilatation of nuclear envelope (Fig. 2D,E). In addition, cytoplasmic polyribosomes disaggregated and degranulation occurred for the membranes of expanded rER; Golgi apparatus was bloated and included large vacuoles (Fig. 2E,F). Nevertheless, the mitochondria appeared normal. The density of cyto-

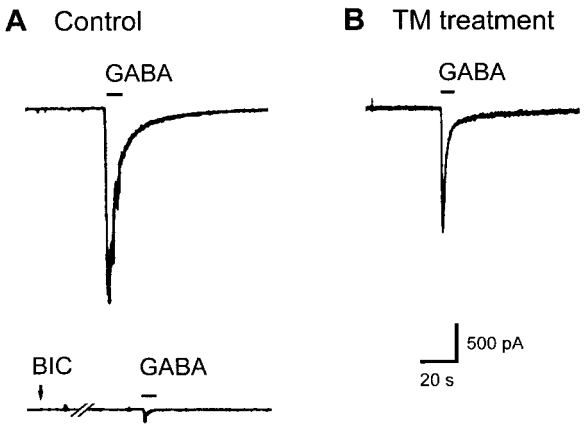

Fig. 1. TM reduces the amplitude of GABA-activated currents in the cultured neurons derived from chicken embryo brains. Whole-cell recordings of GABA-induced currents are shown after rapid topical application of GABA to the untreated control (**A**) and TM-treated (5 μg/ml, 24 h; **B**) neurons at -60 mV. The horizontal bars indicate the duration of the application of 100 μM GABA. The lower trace in A illustrates that the current (in control cells) is blocked intensively by bath application of 20 μM bicuculline, 10 min before the onset of GABA application. The TM treatment decreased about 40% of the control current amplitude.

plasmic and nucleoplasmic matrix appeared somewhat higher in most of the neurons than that of control neurons (Fig. 2D–F).

Neuronal Morphology After Removal of TM

At 3 h following removal of TM (R3h), approximate 10% of the cells detached from the culture dish, and 17% of the remainder incorporated Trypan blue by light microscopic observations [Lin et al., 1998]. Under electron microscopic examination, about 28% (n = 379) of the remaining neurons displayed a lower level of the cytoplasmic matrix density, compared to that of untreated control neurons, and thereby were defined as light neurons (Fig. 3A), whereas the rest were intermediate neurons (Fig. 3B), exhibiting similar cytoplasmic density to control. The cell bodies of approximate 29% (n = 95) of the light neurons were larger in diameter than that of control. The degrees of nuclear indentation and clumping of chromatin appeared lessened in all of the R3h neurons. The rER and nuclear envelope were often dilated and their lumina were filled with flocculent materials. Rosettes of ribosomes reappeared and the regranulation occurred for rER and nuclear envelopes to some extent. About 27% of the neurons contained unusual spindle-like structures (Fig. 3B), which averaged 2.9 ± 0.2 (mean ± standard error) in number per neuron. These spindle-like structures ranged from about 0.5 to 6 μm in length and 0.02 to 1.2 μm in width, and usually had limiting membranes. They were randomly distributed throughout the cytoplasm and a number of them were continuous with the nuclear envelope. In addition, flocculent materials were frequently seen within the spindle-like structure. Cellular debris was present in the extracellular space. However, the mitochondria and Golgi apparatus appeared similar to control (Fig. 3A,B).

For cultures incubated for 24 h after withdrawal of TM (R24h), about 40 to 50% of cells remained attached to the dishes and 47% of these remaining neurons ingested Trypan blue [Lin et al., 1998]. Electron microscopic analysis showed that only about 3% (n = 243) of the neurons attached to culture dish were light neurons, and 15% of these had swollen cell bodies. Approximate 44% of the neurons were dense neurons, which contained many membrane-bound vacuoles, 80 to 600 nm in diameter, in the cytoplasm (Fig. 3D). The remaining 53% of the neurons were intermediate neurons (Fig. 3C). Morphological characteristics of the organelles in most neurons of R24h were similar to that in the neurons of the R3h group, including swelling of rER and nuclear envelope, and the normal appearence of mitochondria and Golgi apparatus. Nevertheless, 88% of the neurons possessed the spindle-like structure, and the mean number of the structure was 9.2 ± 0.8 per neuron, significantly higher than that in the neurons after 3 h recovery. More cellular debris developed in the extracellular space, in comparison with the culture of R3h.

Neuronal Morphology Following Further TM Incubation

For the TM-treated culture (5 μg/ml for 24 h) further exposed to TM for 3 h (TM + 3 h), approximate 29% (n = 284) of the neurons possessed spindle-like structures with a mean of 2.7 ± 0.2 for each neuron. About 45% of the neurons belonged to light neurons and the others were intermediate neurons. For the culture following 24 h additional exposure of TM (TM + 24 h), the percentages became 6%, 60%, and 34% (n = 257), for light, intermediate, and dense neurons, respectively. The spindle-like structures were present in about 81% of the neurons with a mean of 8.3 ± 0.6 per neuron

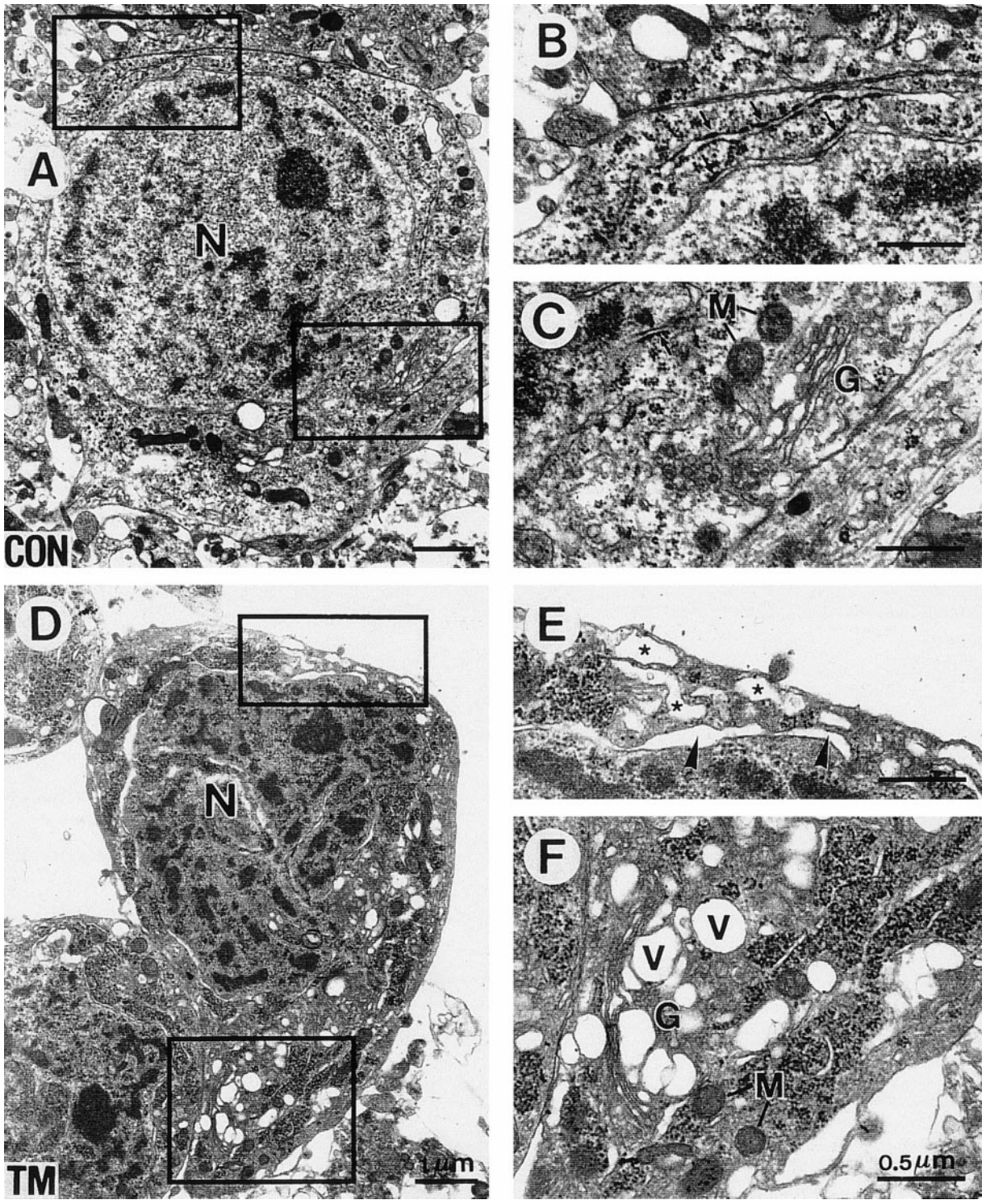

Fig. 2. Electron micrographs illustrating the effect of TM on the ultrastructure of cultured neurons. **A**: An untreated control neuron (CON) with a large nucleus (N). **B,C**: Higher magnifications of boxed areas in the upper left and lower right of A. The ribosomes (arrows) are disseminated in the cytoplasm and also attached to the membranes of rER and nuclear envelope. G, Golgi apparatus. **D**: A neuron subjected to 24 h incubation with 5 μg/ml of TM. The severely indented nucleus (N) contains abundant condensed chromatin. Boxed areas from the top and bottom of D are shown in **E** and **F** at higher magnification to display the distended nuclear envelope (arrow heads), degranulated dilated rER (asterisks), and swollen Golgi apparatus (G) including large vacuoles (V). M, Mitochondrion. Scale bars = 1 μm in A and D; 0.5 μm in B,C,E, and F.

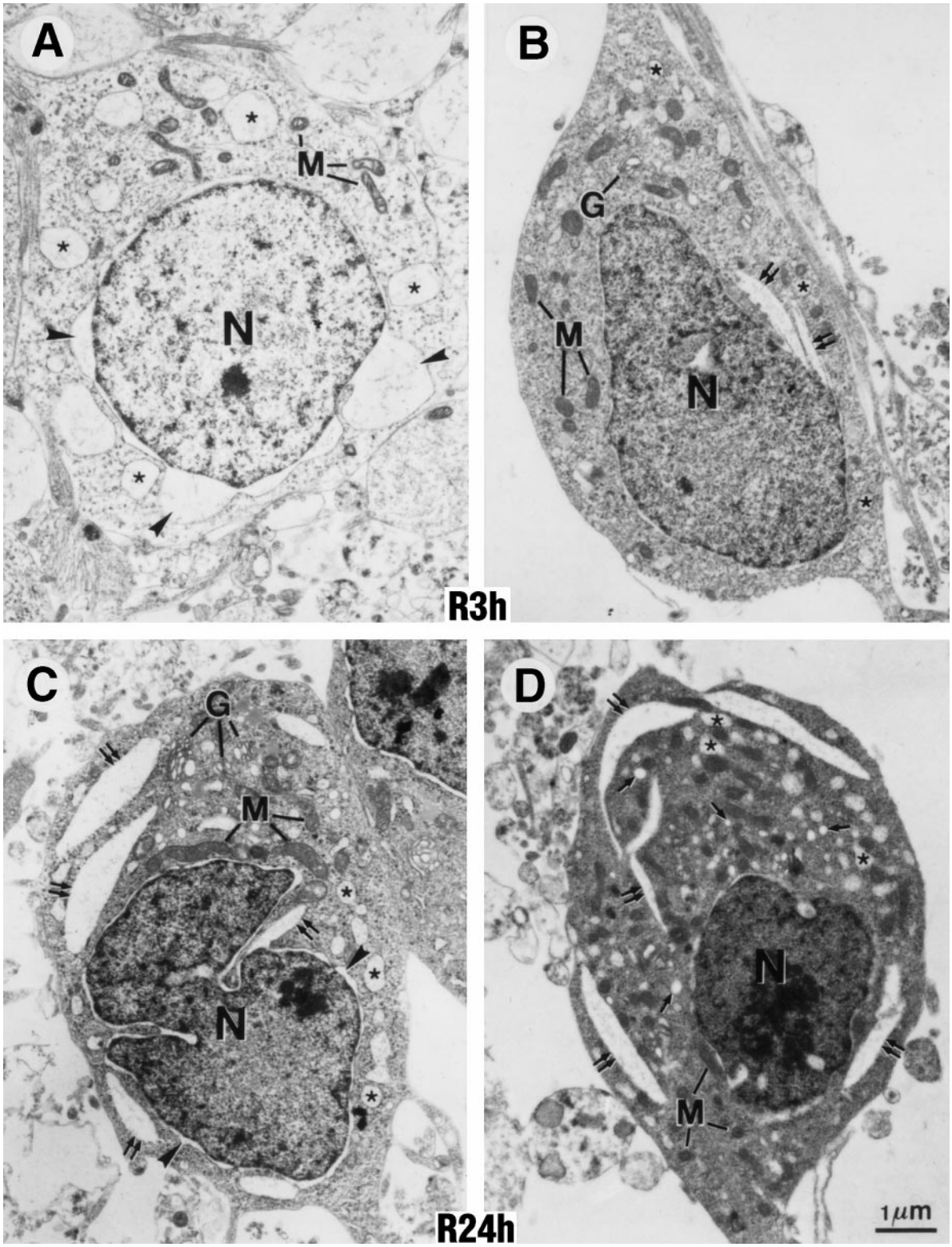

Fig. 3. Degenerating neurons indicating the irreversible effect of TM treatment. Neurons exposed to 5 μg/ml of TM for 24 h were further incubated in fresh medium without TM for 3 h (R3h) or 24 h (R24h). They were arbitrarily classified into 3 categories, the light (**A**), intermediate (**B,C**), and dense (**D**) neurons, based on the level of the density of their cytoplasmic matrix. The rER is expanded in the neurons of A–D (asterisks). The dilated nuclear envelopes (arrow heads) are also seen in A and C. Spindle-like structures (double arrows) are present in the perikarya; a few of them are continuous with the nuclear envelope in B and C. The swollen rER, and nuclear envelope as well as the spindle-like structure apparently contain flocculent materials in their lumina. Many vacuoles (single arrows) are notable in the perikaryon of the dense neuron of D. Cell debris is present extracellularly. The random distribution of clumped chromatin occurred in the nuclei (N) of all neurons. G, Golgi apparatus; M, Mitochondria.

TABLE I. Ultrastructural Characteristics of the Degenerating Neurons Induced by TM Treatment[a]

	Light	Ratio for neurons containing intermediate cytoplasmic matrix	Dense	Ratio for neurons containing spindle-like structure	Mean number of spindle-like structure/neuron
CON	0%	100%	0%	0% (160)	0
TM	0%	100%	0%	0% (179)	0
R3h	28%	72%	0%	27% (379)	2.9 ± 0.2 (39)
R24h	3%	53%	44%	88% (243)	9.2 ± 0.8 (61)
TM + 3h	45%	55%	0%	29% (284)	2.7 ± 0.2 (27)
TM + 24h	6%	60%	34%	81% (257)	8.3 ± 0.6 (44)

[a]Cultured brain neurons prepared from chicken embryos were treated with 5 μg/ml of TM for 24 h (TM), followed by removal of TM and incubation with fresh medium for 3 h or 24 h (R3h or R24h). A number of cultures of the TM group received fresh medium containing TM for additional 3 or 24 h (TM + 3 h, or TM + 24 h). These cultures were fixed with 2.5% glutaraldehyde and subjected to electron microscopic processing as described in Materials and Methods. According to the level of the density of cytoplasmic matrix, neurons were arbitrarily classified into light, intermediate, and dense neurons. Data include percentages of neurons in respective groups and the frequency of the spindle-like structure in neurons containing the structure is presented as the mean number ± standard error/neuron. The numbers in parentheses are the number of counted neurons. CON, Untreated control.

(Table 1). The neurons of TM + 3 h and TM + 24 h groups still expressed a certain degree of ribosomal reaggregation and reattachment to rER membranes, dilatation of rER and nuclear envelope, and seemingly normal appearance of mitochondria and Golgi apparatus, in resemblance with that of the R3h and R24h groups. The phenomenon of cytoplasmic vacuolation was also noted in the dense neurons. About 32% and 13% of the light neurons were swollen in the TM + 3 h and TM + 24 h.

Gel Electrophoresis

To determine whether TM administration resulted in DNA fragmentation, which is considered to be one of the characteristics of most apoptotic processes, DNA samples from the neuronal cultures were subjected to agarose gel electrophoresis. After electrophoresis, DNA laddering was absent on the gels for untreated control or various groups of the agent-treated neurons, indicating that TM failed to induce DNA fragmentation in the cultures (Fig. 4).

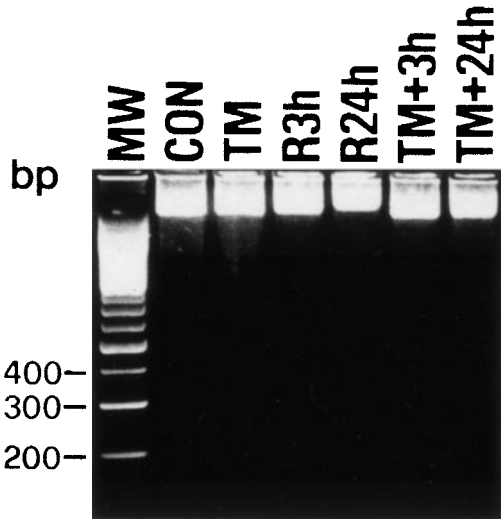

Fig. 4. Photograph of gel electrophoresis illustrating the absence of DNA fragmentation in the untreated control (CON) and TM-treated neuron cultures. The cultures were treated with 5 μg/ml of TM for 24 h (TM) followed by further incubation in fresh medium with (TM + 3 h; TM + 24 h) or without (R3h; R24h) 5 μg/ml of TM for 3 or 24 h. Agarose gel electrophoresis of the DNAs extracted from the cultures was performed as depicted in Materials and Methods. A 100 bp-ladder was used as molecular weight marker (MW). This result is representative of five experiments.

DISCUSSION

The present report examines the mechanism underlying neuronal death in culture induced by TM. Our main findings concern irreversibility of TM toxicity and variance in ultrastructural changes among the degenerating neurons.

N-Glycosylation and Neuronal Ultrastructure

TM is thought to act at rER to block N-linked glycosylation of nascent glycoproteins. In this study, exposure of the cultured neurons to 5 μg/ml of TM for 24 h caused the expansion of nuclear envelope, rER, and Golgi apparatus, as shown by electron microscopic analysis. These changes may correlate with our previous biochemical results, in which the TM treatment inhibited the incorporation of ^{3}H-mannose and ^{3}H-galactose by the cultures [Lin et al., 1998].

Because mannose and galactose are added to glycoproteins in rER and Golgi apparatus respectively [Kornfeld and Kornfeld, 1985], the TM-induced distention of these organelles might be due to the accumulation of the precursors of N-linked glycoproteins within the organelles, in addition to other possible mechanisms. The TM-treated leukemic and endothelial cells showed similar changes in their rER; the accumulation of certain proteins was implicated in the rER [Morin et al., 1983; Tiganis et al., 1992]. Furthermore, disruption of rER and Golgi apparatus were seen in neurons of mouse cerebellar explants following 10 day incubation of TM [Carroll and Bird, 1991].

Irreversible Neuronal Degeneration

Regardless of the inclusion of TM in the prolonged incubation, the morphological features of degenerating neurons and the amount of extracellular cell debris increased with time of the incubation. The presence of the unusual spindle-like structure in the perikarya could also be one of the indications for continual deterioration of cell viability, because it only appeared in the further incubation period, and the ratio of cells containing the structures was elevated with time of incubation. However, the neurons seemed to express some recovering ability by the reappearance of rER-attached ribosomes and cytoplasmic ribosomal rosettes.

We have grouped the neurons into three categories based on the degree of density of cytoplasmic matrix. The ratio of light neurons decreased from 28% to 3% during the 3 h (R3h) to 24 h (R24h) recovery without TM. This probably points to that the majority of these neurons may have died from mechanisms related to necrosis, as most of the morphological features they displayed resemble that of necrosis. For the neurons of prolonged 3 h incubation with TM, the presence of a greater ratio of light cells, 45% than 28% of that without TM, possibly indicate that the further inclusion of TM generates a severer effect.

The dense neurons were not noted in the R3h and TM + 3 h groups, but appeared as 44% and 34% in the R24h and TM + 24 h groups. Though condensed cytoplasm in these neurons corresponds to one of the features of apoptosis, they did not have the apoptotic bodies, neither exhibited margination of clumped chromatin in the nucleus. Furthermore, another criterion of apoptosis, DNA fragmentation was absent in the cultures treated with TM. Similar morphological characteristics have been described for cultured cortical neurons exposed to kainate or AMPA together with dizocilpine maleate (MK-801) and degenerating neurons of newborn rat after the injection of GluR agonists [Regan et al., 1995; Portera-Cailliau et al., 1997]. It is possible that these dense neurons represent another form of degeneration. Because the culture of our study was prepared from the entire brain of chicken embryo, various types of neurons may respond differentially to the perturbation of TM and thereby express different degeneration characteristics. Parallel phenomenon was reported for GluR agonist-elicited neuronal death in vivo; the type of mechanism for cell death depended on the distinct brain areas and age of animal [Portera-Cailliau et al., 1997].

It is likely that the intermediate neurons are in a less severe state of degeneration, since their cytoplasmic density is similar to control. Previous Trypan blue incorporation experiment has shown that about 17% and 47% of cells of R3h and R24h are degenerating [Lin et al., 1998]. Nonetheless, the remaining 83% and 53% surviving neurons are close to the 72% and 53% of intermediate neurons seen in the present report. This could also support the notion that they are healthier. Whether these neurons will transform to light or dense neurons awaits more study.

Apoptosis has been claimed the mechanism for the death of cultured sympathetic neurons, cerebellar granule cells, and non-neuronal cell lines, because DNA fragmentation or the inhibition of cell death by cycloheximide was detected, in addition to light microscopic morphological evidence [Pérez-Sala and Mollinedo, 1995; Carlberg et al., 1996; Chang and Korolev, 1996; Chang et al., 1997; Dricu et al., 1997]. By contrast, the criterion of DNA fragmentation does not seem to apply for apoptosis in several experiments, since which have found that DNA fragmentation is absent for neurons exhibiting ultrastructural characteristics of apoptosis, and is present in necrotic neurons [Bøe et al., 1991; Cohen et al., 1992; Van Lookeren Campagne et al., 1995].

Our previous study has shown a 13% decrease in protein synthesis, but 44% to 91% blockade to glycosylation of neurons treated with TM for 24 h [Lin et al., 1998]. It is apparent that the impaired glycosylation generated by TM causes an extensive malfunction of the

neurons, and the toxicity is irreversible. Whether mechanisms underlying the neuronal death relate to the genes that involve apoptosis, such as p53, Bax, and Bcl-2 [Bredesen, 1995], requires further investigations.

The Spindle-Like Structure in TM-Treated Neurons

Analogous to the spindle-like organelle in our study, the cytoplasmic slashes and clear spindles, have been described in epithelial cells of secretory tubule in the Harderian gland of the Mongolian gerbil (*Meriones unguiculatus*) [Johnston et al., 1983] and in the pineal gland of the yellow-bellied country rat (*Rattus losea Swinhoe*) [Huang et al., 1989], respectively. The clear spindles in the pineal gland have been proposed to be secondary lysosomes that carry out focal degradation, based on the presence of acid phosphatase activity in this structure, as revealed by electron microscopic histochemistry [Huang et al., 1989]. We propose that the spindle-like structure in our study might participate in clearing degenerating compartments, for the number of the structure is increased with time. However, the significance of the spindle-like structure demands additional examinations including electron microscopic histochemistry.

N-Glycosylation and Electrophysiology of $GABA_AR$

In the present study, electrophysiological recordings revealed that the amplitude of GABA-elicited currents was blocked by 40% in the neurons after 24 h incubation of 5 μg/ml of TM. Similarly, a nearly complete inhibition of GABA-evoked currents was observed in TM-treated *Xenopus* oocytes injected with the mRNAs of rat brain or chick optic lobe [Sumikawa et al., 1988]. It has been reported earlier that TM reduces the expression of Na^+-K^+-pump in cultured skeletal muscle [Alboim et al., 1992]. Besides affecting the $GABA_AR$, TM could likewise alter the expression of other membrane glycoproteins in the neurons, such as the Na^+-K^+-pump and other types of ion channels, resulting in interrupted ion homeostasis, and thus leading to neuronal death.

The decrease of 40% in the currents is larger than the decreases of 20% and 28% in the binding levels of ^{3}H-flunitrazepam and ^{3}H-muscimol shown in our previous study using the same preparation [Lin et al., 1998]. This discrepancy might be caused by that the electrophysiological analysis revealed the downregulated function of $GABA_AR$ on the cell membrane, whereas receptor binding assay disclosed changes in the total number of intracellular and cell surface receptors. The decreased functional expression of $GABA_AR$ could reflect the deteriorated neuronal viability, in accordance with the morphological degeneration.

In conclusion, chronic TM incubation rendered irreversible degeneration of cultured embryonic brain neurons, which was manifested by differential ultrastructural traits. Different populations of the degenerating neurons seem to undergo necrosis, and other distinct mechanisms from apoptosis for cell death. The data indicate that N-glycosylation of proteins is important for the maintenance of the normal expression of $GABA_AR$ and crucial for neuronal survival.

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