—Regular Article—

Higher Toxicity of Dibutyltin and Poly-L-Lactide with a Large Amount of Tin but Lower Toxicity of Poly-L-Lactide of Synthetic Artificial Dura Mater Exhibited on Murine Astrocyte Cell Line

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Neurotoxicities of dibutyltin (DBT), tin(II) octylate (OT), poly-L-lactides (PLLA, molecular weight [MW] = 5000, PLLA 5000), PLLA without tin (MW = 3000, PLLA 3000), PLLA with a large amount (590 ppm) of tin (S3), poly(glycolic acid-co-ε-caprolactone) oligomer (MW = 6200, PGC oligomer), and poly(L-lactic acid-co-glycolic acid-co-ε-caprolactone) oligomer (MW = 6400, PLGC oligomer) related to artificial dura mater were examined using the murine astrocyte cell line, CRL-2534. The indices were cell viability, glutamate concentration in the cell supernatant, and cell proliferation. Lower cell viability was observed among cells exposed to 0.5 μM DBT or 10 μg/ml of S3. There were no differences in cell viability of astrocytes exposed to OT, PLLA 5000, PLLA 3000, PGC oligomer, or PLGC oligomer. Mean glutamate concentration in the supernatant of cells exposed to 0.25 μM DBT was higher than that of the control after 2 h incubation. Lower mean concentration of glutamate in the supernatant of cells exposed to 5 μg/ml of S3 was observed after 2 h incubation. Cells exposed to 50 μg/ml of PGC oligomer had a higher mean concentration of glutamate in the supernatant. OT only inhibited cell proliferation at 100 μM. Proliferation of cells exposed to 0.25 μM or 0.5 μM DBT was inhibited, as was that of cells exposed to 100 μM OT, 50 μg/ml PLLA 5000, 50 μg/ml PLLA 3000, and 5 μg/ml S3, 5 d and 7 d after exposure. Although DBT does not reach levels that induced neurotoxicity in artificial dura mater, these results suggest that DBT is neurotoxic and PLLA toxicity increases with the increase in tin concentration.

Key words——neurotoxicity; dibutyltin; astrocyte; artificial dura mater; poly-L-lactide; tin(II) octylate

INTRODUCTION

A synthetic biodegradable artificial dura mater that is mainly composed of poly-L-lactides (PLLA) has been developed.[1] The artificial dura mater, which is applied to the surface of the brain after brain surgery, usually has a three-layer structure in which a gel-like form is inserted between two elastic structures. After several months, it is absorbed, making repeat surgery for removal unnecessary. In addition, the risk of infection from an allograft or xenograft can thus be avoided.[2]

Catalysts including dibutyltin (DBT)[3–5] and tin (II) octylate (OT)[6] are used in the synthesis of PLLA polymers, and these tin compounds remain in the artificial dura mater after the synthesis. The major toxic effects of organotins are immunotoxicity and neurotoxicity. The immunotoxicity of DBT has been reported previously.[5,7,8] Because permiability of DBT across the blood brain barrier is not high due to its relatively high polarity,[3,5] the central nervous system is not exposed to very much DBT from oral exposure. However, when an artificial dura mater is clinically applied, the brain tissue is directly exposed to DBT, which may also have cytotoxic effects on the cells in the central nervous system.

Tributyltin (TBT), which is neurotoxic,[4,5,9] is degraded to DBT in the environment and in organisms.[5] DBT was detected in the brains of rats after acute oral administration of TBT.[3] The neurotoxicity of TBT may be partly due to DBT, which is metabolized in the brain. Therefore, the evaluation of the neurotoxic

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effects of DBT is important.

The neurotoxicity of OT has not been reported. Because the brain is also exposed to OT during the absorption of artificial dura mater, it is necessary to evaluate the neurotoxicity of OT. In addition, an artificial dura mater is usually composed of not only PLLA but also glycolic acid and caprolactone. It is, therefore, also necessary to evaluate the toxicities of OT and these materials.

To examine whether or not PLLA and tin compounds have synergetic toxicities, we conjectured that a comparison of an artificial dura mater, PLLA without tin compounds, and PLLA containing a high level of tin would be useful. Moreover, there could be possible changes in toxicity as the artificial dura mater is degraded. The difference in the neurotoxicity of the poly(glycolic acid-co-ε-caprolactone) oligomer (PGC oligomer) and poly(L-lactic acid-co-glycolic acid-co-ε-caprolactone) oligomer, which maintains its elastic structure (PLGC oligomer), should also be clarified.

Even though the assessment of the safety of artificial dura mater is needed before clinical application, a simple and easy method for safety assessment of toxicants of the central nervous system has not yet been established. However, the evaluation of the toxic effect of a component on a cell line is generally useful to assess its safety simply for screening, and it does not necessitate killing experimental animals. Cell viability is usually used as an index for the evaluation of cytotoxic effects on a cell line.[10–12] Astrocytes are one of the components of brain cells. A murine astrocyte cell line, CRL-2534, astrocyte type III[13,14] is commercially available. CRL-2534 cells function as do astrocytes, which metabolize glutamate to glutamine and discharge metabolized glutamine outside the cell.[14] Although astrocytes are not neuronal cells, they are part of the central nervous system and play a role in metabolism in the brain. Therefore, the use of CRL-2534 may be helpful to evaluate toxic effects on the central nervous system. The determination of the glutamine concentration in the supernatant of CRL-2534 cells can be used as an index of cell function.

In addition to the cell viability, the effect of a component on cell proliferation is also a sensitive index of cell toxicity. Exposure to a chemical at a level lower than that causes cell death may inhibit cell proliferation.

In the present study, the toxic effects of the following materials on CRL-2534 cells were examined: DBT, OT, PLLA without tin (PLLA 3000), poly-L-lactic acid 5000 with $<$10 ppm tin (PLLA 5000), PLLA with 590 ppm tin (S3), PGC oligomer, and PLGC oligomer. The indices for the evaluation were cell viability, the release of glutamate from the cells, and the effects on cell proliferation. The aim of the current study was the evaluation of the cytotoxic effects of DBT, OT, various PLLAs, and two oligomers *in vitro* by using the murine astrocyte-lineage cell line, CRL-2534.

MATERIALS AND METHODS

Samples and Cell Line The materials examined in this study were: DBT dichloride (Wako, Osaka), OT (Wako), PLLA 5000 (molecular weight [MW] $=$5000, tin concentration $<$10 ppm, Nacalai Tesque, Inc., Kyoto) as artificial dura mater, PLLA 3000 (Taki Chemical, Kakogawa, MW$=$3000, tin concentration 0 ppm), and S3, with a large amount of tin (Taki Chemical, MW$=$11000, tin concentration 590 ppm), PGC oligomer (MW$=$6200, tin concentration $<$20 ppm), and PLGC oligomer (MW$=$6400, tin concentration $<$20 ppm, Kawasumi Laboratories, Inc., Tokyo). PLLA 3000, S3, PGC oligomer, and PLGC oligomer were made in the laboratories. As the cell line, the murine astrocyte cell line, CRL-2534, astrocyte type III (The American Type Culture Collection, Manassas, VA, USA) was used.

Cell Culture Conditions CRL-2534 cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) containing 75 U/ml penicillin and 75 μg/ml streptomycin (Meiji Seika, Tokyo) with 10% fetal bovine serum (HyClone, Logan, UT, USA) in a humidified incubator at 37°C with 5% CO_2. For each experiment there were: 2.5×10^5 cells/well in a 24-well cell culture plate for cell viability, 1.0×10^6 cells/well in a 6-well cell culture plate for discharge of glutamate into culture medium, and 1.0×10^4 cells/well in a 24-well cell culture plate for cell proliferation. Before all experiments, the cell viability was checked and was always 90% or higher.

Cell Viability The concentrations of DBT used were determined by following the previous studies for DBT[11] and TBT.[10] CRL-2534 cells were exposed to DBT dissolved in ethanol at the concentrations of 0, 0.125, 0.25, and 0.5 μM in 24-well cell culture plates and incubated for 24 h. The concentrations of OT, PLLAs, PGC oligomer, and PLGC oligomer tested were decided by preliminary experiments. CRL-2534

cells were exposed to OT dissolved in ethanol at the concentrations of 0, 25, 50, 75, and 100 μM in 24-well cell culture plates. The final concentration of ethanol for both DBT and OT exposure was 0.1%. CRL-2534 cells were exposed to PLLA, PGC oligomer, and PLGC oligomer except for S3 at the concentrations of 0, 10, 20, and 50 μg/ml in 24-well cell culture plates. CRL-2534 cells were exposed to S3 at 0, 2.5, 5, and 10 μg/ml in the plates. PLLAs, first dispersed in dimethylsulfoxide (DMSO, Nacalai Tesque) were dispersed in the culture medium by sonication. The final concentration of DMSO in each well was 0.5%.

The cell viability of CRL-2534 cells exposed to tested samples was evaluated by the trypan blue exclusion test. Because the processes of CRL-2534 cells were easily detached at the cell sampling, 0.05% trypsin-EDTA (Invitrogen) was used for the cell sampling from each well to avoid detachment of the processes from the CRL-2534 cells.

In addition, the cytotoxicity was also evaluated by lactic acid dehydrogenase (LDH) in the supernatant. After incubation for 24 h, part of the supernatant in each well was sampled for the LDH measurement. LDH was measured by a spectrophotometer (Tosoh Corp., Tokyo) at the wavelength of 492 nm using CytoTox 96 (Promega, Madison, WI, USA).

Glutamate Concentration in Supernatant CRL-2534 cells were exposed to the concentrations of 0 and 0.25 μM DBT and 0 and 100 μM OT dissolved in ethanol in 6-well cell culture plates. Other CRL-2534 cells were exposed to PLLA 5000, PLLA 3000, PGC oligomer, and PLGC oligomer at the concentrations of 0 and 50 μg/ml in 6-well cell culture plates. In addition, the glutamate levels of cells exposed to 5 μg/ml of S3 were also determined.

The glutamate concentrations in the cell supernatant were determined based on the method of Aoyagi and Takahashi.[15] For DBT and S3, the supernatant was sampled immediately after the exposure and again 1 h and 2 h later. For OT, PLLA 5000, PLLA 3000, PGC oligomer, and PLGC oligomer, the supernatant was sampled immediately after the exposure and again 3 h and 6 h later. Before sampling, each well was washed twice with a large amount of Dulbecco's phosphate buffered saline (PBS, Sigma, St. Louis, MO, USA) at 37°C, and washed at least three times with 1 ml of PBS. Three minutes after the last addition of 1 ml of PBS, the supernatant was sampled. The samples were filtered through a Cosmonice Filter W (pore size 0.45 μm) (Nacalai Tesque). The concentration of glutamate in the sample was determined by reverse-phase HPLC with a Crestpak C18 column (4.6×150 mm) (Jasco, Tokyo) at a column temperature of 20°C. The mobile phase used from the start of analysis to 8 min consisted of Buffer A (50 mM CH3COONa, pH 6.0): Buffer B (HPLC-grade methanol : tetrahydrofruan = 9 : 1) = 2 : 1. Buffer B alone was used for the mobile phase from 8 min to 16 min. The mobile phase of Buffer A : B = 2 : 1 was used again from 16 min to 20 min. The flow rate was 1.0 ml/min for the analysis. When each sample was injected by an autosampler, *o*-phthalaldehyde (Wako Chemical Co., Tokyo) was added as a fluorescent reagent. We used a Jasco FP-920 Fluorescence Detector (Jasco).

Cell Proliferation CRL-2534 cells were exposed to DBT dissolved in ethanol at the concentrations of 0, 0.25, and 0.5 μM in 24-well cell culture plates. The cells were exposed to OT dissolved in ethanol at the concentrations of 0 and 100 μM in 24-well cell culture plates. CRL-2534 cells were exposed to 50 μg/ml of PLLA 5000, PLLA 3000, PGC oligomer, or PLGC oligomer, and 5 μg/ml of S3.

The cell proliferation was examined by counting the number of cells and the uptake of the TetraColor ONE (Seikagaku Corp., Tokyo). TetraColor ONE was added to the cells at days 1, 3, 5, and 7. After adding TetraColor ONE, the cells were incubated for 2 h at 37°C, and the supernatant was sampled. The concentration of formazan, a metabolite of TetraColor ONE, in the supernatant was determined at the wavelength of 450 nm using a spectrophotometer. Cell numbers and cell viability were simultaneously determined by the trypan blue exclusion test.

Statistical Analyses The mean values of cell viability and LDH levels (% control) were calculated. The mean values of glutamate concentrations or formazan concentrations were also calculated. The results were compared by ANOVA (one-way analysis of variance), Fisher's protected least significant difference (PLSD) test as a post hoc test among the three groups, and by the Student's *t*-test or Mann-Whitney's U test between each of the two groups, followed by whether or not the F test between the groups was significant. Statistical software used was Statview version 5.0 (SAS Institute Inc., Cary, NC, USA).

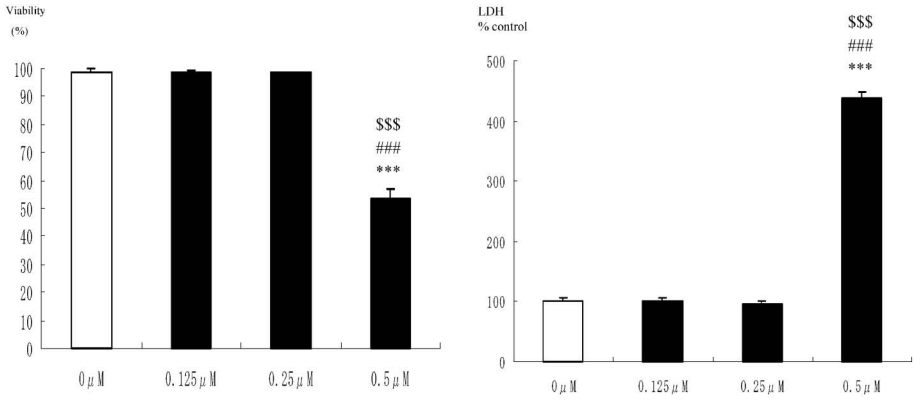
RESULTS

Cell Viability Figure 1 illustrates the cell viability (A) and LDH levels (% control) in the supernatant (B) of astrocytes exposed to 0, 0.125, 0.25, and 0.5 μM DBT. The mean values of cell viability in the groups of astrocytes exposed to 0.5 μM DBT were significantly lower than those in the control group and the other treated groups. The mean values of LDH levels in the supernatant (% control) in 0.5-μM DBT-exposed groups were significantly higher than those in the control group and the other treated groups.

There were no significant differences in the mean values of cell viability or LDH (% control) among the cells exposed to OT, PLLA 5000, or PLLA 3000. Figure 2 illustrates the cell viability (A) and LDH (% control) in the supernatant (B) in the astrocytes exposed to 0, 2.5, 5, and 10 μg/ml S3. The mean value of cell viability in the 10-μg/ml S3-exposed group was significantly lower than that in the control group and the other S3-exposed groups. The mean LDH (% control) in the 10-μg/ml S3-exposed group was significantly higher than those in the control group and the DBT-exposed groups. The mean cell viability and LDH concentrations in the cells exposed to PGC oligomer or PLGC oligomer did not show any significant differences compared to the control group.

Glutamate Concentration in the Supernatant Figure 3 illustrates glutamate concentrations in the supernatant of the cells exposed to DBT at 0 and 0.25 μM for 1 h (A) and 2 h (B). At 1 h, the mean glutamate concentration in the DBT-exposed group was not significantly different compared to the control group, but at 2 h, the mean glutamate concentration in the DBT-exposed group was significantly higher than that in the control group. The mean glutamate concentrations in the groups exposed to OT, PLLA 5000, and PLLA 3000 did not show any significant differences compared to the control group at 3 h and 6 h (mean glutamate concentration [(μM)] $\pm$ standard error [S.E.] were; OT: 0.123$\pm$0.032 at 3 h, 0.208$\pm$0.048 at 6 h; PLLA5000: 0.188$\pm$0.020 at 3 h, 0.214$\pm$0.012 at 6 h; PLLA3000: 0.130$\pm$0.018 at 3 h, 0.214$\pm$0.012 at 6 h). Figure 4 illustrates glutamate concentrations in the supernatant of astrocytes exposed to S3 at 0 and 5 μg/ml for 1 h (A) and 2 h (B). At 1 h, the mean glutamate concentration in the S3-exposed group was not significantly different compared to the control group. However, at 2 h, the mean glutamate concentration in the S3-exposed group was significantly lower than that in the control group.

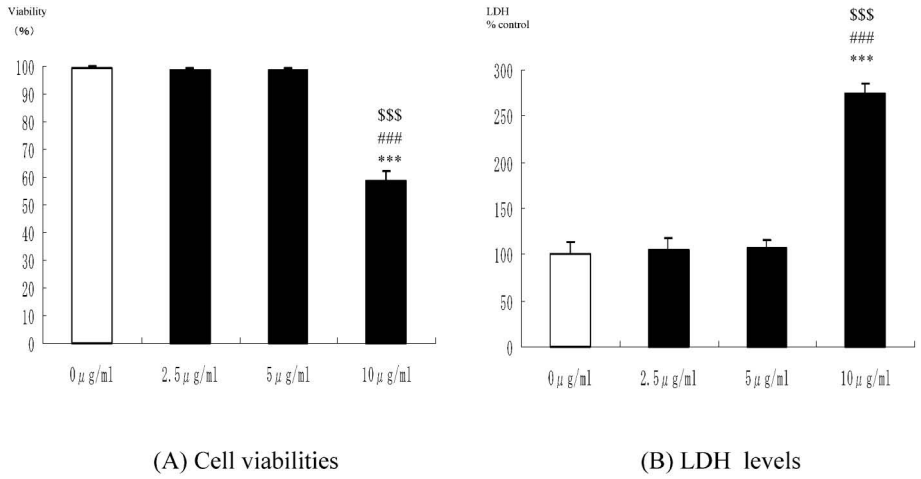
Figure 5 illustrates the glutamate concentrations in the supernatant of astrocytes exposed to PGC oligomer at 3 h (A) and 6 h (B) or PLGC oligomer at 3 h (C) and 6 h (D). The mean glutamate concentrations in the PGC-oligomer-exposed groups were significantly higher at both 3 h and 6 h than those in the respective control groups. For the groups exposed to PLGC oligomer, the mean glutamate concentration at 3 h was not significantly different compared to that



(A) Cell viabilities (B) LDH levels

Fig. 1. Viability and LDH Levels in the Supernatant of Astrocytes Exposed to DBT Dichloride after 24 h Incubation

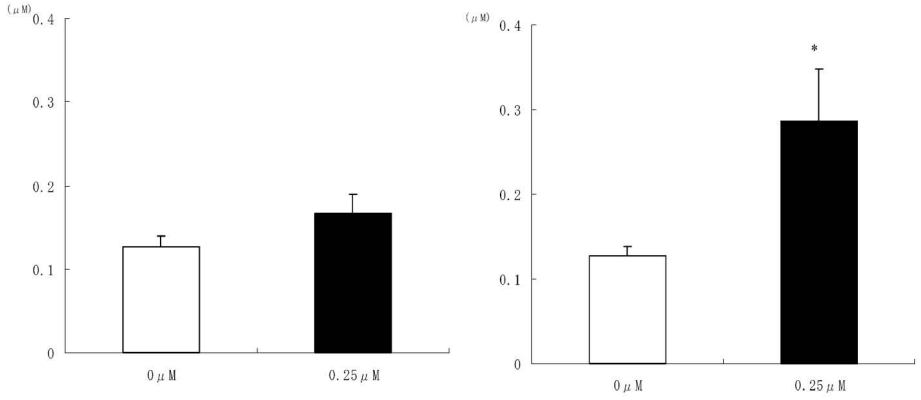
A. The viability of CRL-2534 cells exposed to DBT at 0–0.5 μM. B. The levels of LDH in the supernatant of CRL-2534 cells exposed to DBT at 0–0.5 μM. Viability was determined by trypan blue exclusion test. LDH level was measured by a spectrophotometer at wavelength 492 nm and expressed as % control. Means$\pm$standard errors are indicated (n=6); $^{***}p<0.001$ compared with the 0 μM group; $^{\#\#\#}p<0.001$ compared to the 0.125 μM group; $^{\$\$\$}p<0.001$ compared with the 0.25 μM group by Fisher's PLSD test.



(A) Cell viabilities (B) LDH levels

Fig. 2. Viability and LDH Concentrations in Supernatant of Astrocytes Exposed to S3 after 24 h Incubation

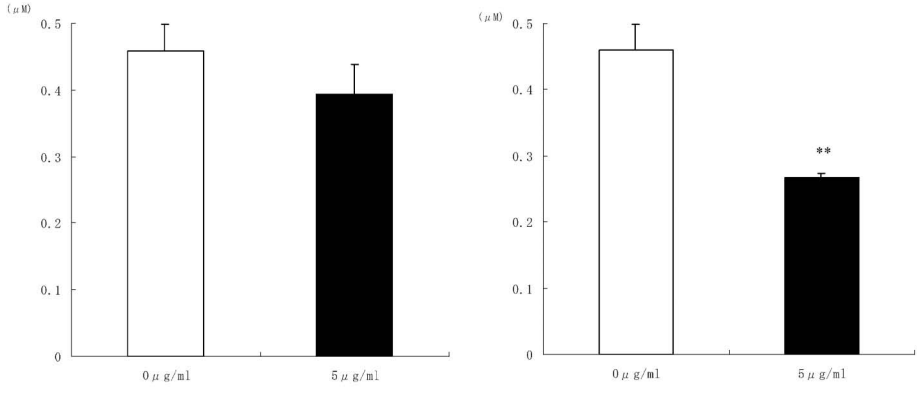
Means±standard errors are indicated (n=6); $^{***}p<0.001$ compared with the 0 μg/ml group; $^{\#\#\#}p<0.001$ compared with the 2.5 μg/ml group; $^{\$\$\$}p<0.001$ compared with the 5.0 μg/ml group by Fisher's PLSD test.



(A) 1 h incubation (B) 2 h incubation

Fig. 3. Glutamate Concentrations in Supernatant of Astrocytes Exposed to DBT Dichloride after 1 h or 2 h Incubation

Means±standard errors are indicated (n=4); $^{*}p<0.05$ compared to the 0 μg/ml group by Student's t-test (A) and Mann-Whitney's U test (B).



(A) 1 h incubation (B) 2 h incubation

Fig. 4. Glutamate Concentrations in Supernatant of Astrocytes Exposed to S3 after 1 h or 2 h Incubation

Means±standard errors are indicated (n=4); $^{**}p<0.01$ compared with the 0 μg/ml group by Student's t-test (A) and Mann-Whitney's U test (B).

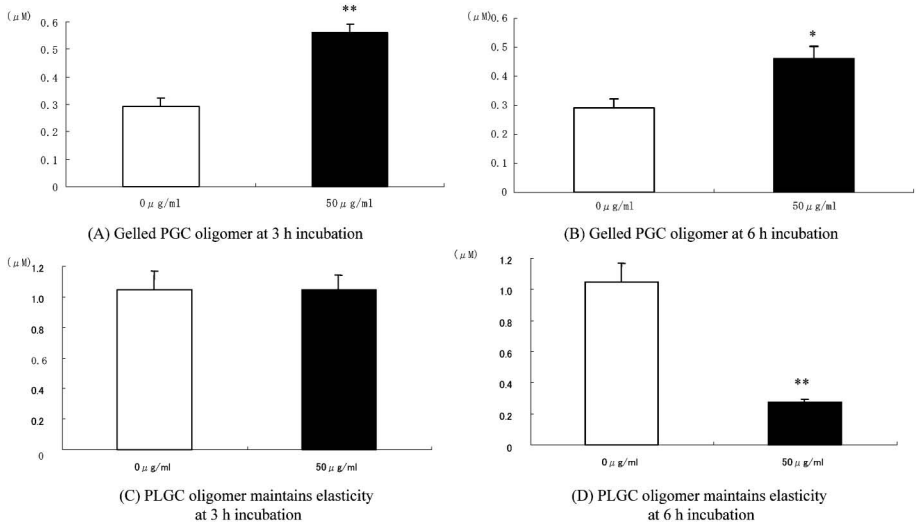


Fig. 5. Glutamate Concentrations in Supernatant of Astrocytes Exposed to PGC Oligomer or PLGC Oligomer, Which Maintain Elasticity after 3 h or 6 h Incubation

Means±standard errors are indicated ($n=4$), *$p<0.05$, **$p<0.01$ compared with the 0 μg/ml group by Student's t-test.

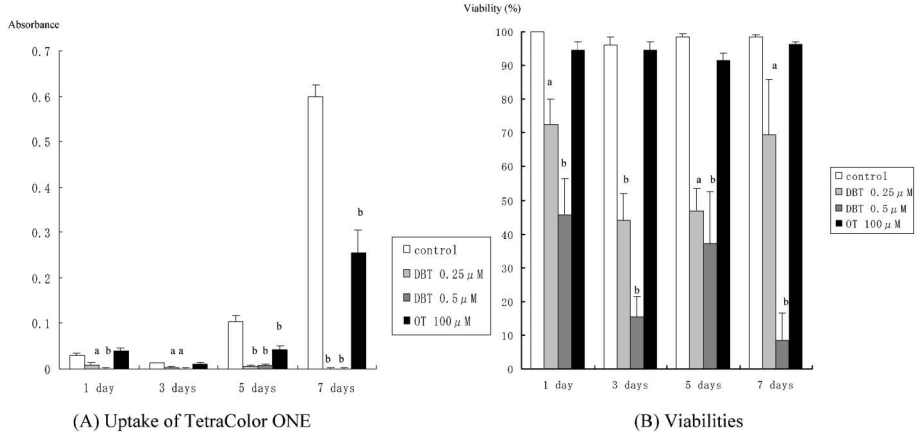


Fig. 6. Uptake of TetraColor ONE and Viability of Astrocytes Exposed to DBT Dichloride or OT

Means±standard errors are indicated ($n=6$); a, $p<0.01$; b, $p<0.001$ compared with the control group by Fisher's PLSD test.

in the control group. At 6 h, the mean glutamate concentration was significantly lower than that in the control group.

Cell Proliferation Figure 6 illustrates the results of the TetraColor ONE inclusion test (A) and cell viability (B) for the evaluation of the proliferation of astrocytes exposed to DBT or OT. The mean formazan (a metabolite of TetraColor ONE) levels in the supernatant and mean cell viability in all DBT-exposed groups were significantly lower than those of the respective control groups at all observed time points. The mean formazan in the OT-exposed groups were significantly lower than those in the respective control groups at 5 d and 7 d after the exposure. While, there was no significant difference in the cell viability between each OT-exposed group and the respective control. The results of the TetraColor ONE inclusion test (A) and viability (B) for astrocytes exposed to 50 μg/ml of PLLA 5000, PLLA 3000, 5 μg/ml of S3, and 50 μg/ml of PGC oligomer and PLGC oligomer are shown in Table 1. The mean formazan levels or cell viability in the S3-exposed groups at 5 d and 7 d were significantly lower than those in the other groups. The formazan levels in the supernatant of the PLLA 5000- and PLLA 3000-exposed groups at 7 d were significantly lower than those in the respective controls. No significant differences in the formazan concentrations or the cell via-

Table 1. TetraColor ONE Uptake and Viabilities of Astrocytes Exposed to PLLA 5000, PLLA 3000, S3, PGC Oligomer or PLGC Oligomer, Which Maintains Elasticity

(A) TetraColor ONE Uptake (Formazan Concentration)

	1 d	3 d	5 d	7 d
Control	0.103±0.003	0.101±0.005	0.151±0.001	0.333±0.003
PLLA5000 50 μg/ml	0.111±0.004	0.092±0.003	0.153±0.002	0.307±0.004[b]
PLLA3000 50 μg/ml	0.105±0.007	0.095±0.004	0.151±0.002	0.298±0.006[c]
S3 5 μg/ml	0.106±0.005	0.092±0.004	0.139±0.002[a]	0.261±0.005[a]
PGC oligomer 50 μg/ml	0.111±0.004	0.099±0.002	0.151±0.001	0.333±0.004
PLGC oligomer 50 μg/ml	0.111±0.004	0.103±0.004	0.150±0.001	0.323±0.005

Means±standard errors (absorbance at 450 nm) are indicated ($n=6$).

(B) Viabilities

	1 d	3 d	5 d	7 d
Control	99.0±1.0	99.1±0.6	99.2±0.4	99.6±0.2
PLLA5000 50 μg/ml	97.1±2.9	99.0±0.6	97.9±0.4	99.4±0.2
PLLA3000 50 μg/ml	99.0±1.0	98.4±1.1	98.7±0.5	99.6±0.1
S3 5 μg/ml	96.2±2.5	97.9±1.0	95.3±0.7[a]	97.5±0.4[a]
PGC oligomer 50 μg/ml	95.8±2.6	99.5±0.5	98.8±0.6	99.4±0.3
PLGC oligomer 50 μg/ml	97.1±1.8	99.5±0.5	98.4±0.3	99.6±0.2

Means±standard errors are indicated ($n=6$). [a] $p<0.001$ compared to the other groups by Fisher's PLSD test, [b] $p<0.001$ compared to control and PGC oligomer by Fisher's PLSD test and $p<0.05$ compared to the PLGC oligomer by Fisher's PLSD test, [c] $p<0.001$ compared to control, PGC oligomer, and PLGC oligomer by Fisher's PLSD test.

bility were observed compared to the control in the group exposed to PGC oligomer or PLGC oligomer.

DISCUSSION

The use of a cell line to evaluate toxicity is useful especially for its simplicity and does not necessitate killing experimental animals. The cell line, CRL-2534, murine astrocyte type III, which was derived from murine cerebellum cells at 8 months of age and naturally transformed, have become available.[13] CRL-2534 cells metabolize glutamate to glutamine, and discharge glutamine outside the cells.[14] Astrocytes also take up and metabolize neurotransmitters including glutamate.[6] Glutamate receptors are the most ubiquitous receptors expressed in the astrocytes and closely related to the functions of astrocytes. In a study by Matthias *et al.*,[17] the two types of astrocytes in the murine hippocampus, cells that expressed glutamate receptors, and cells that possessed glutamate transport currents were observed. The use of CRL-2534, as a part of the central nervous system, may prove useful to evaluate cytotoxicity in the central nervous system.

In the present study, the exposure levels of the astrocyte cell line, CRL-2534, to DBT were based on previous studies of the macrophage cell line, J774.1, for DBT[11,12] and TBT toxicities.[10] For OT, the exposure levels were set to be relatively higher than those of DBT because of the higher concentration used as a catalyst, the absence of previous neurotoxicity reports, and the results of preliminary experiments. PLLA 5000, PLLA 3000, and S3 were compared at the levels established in preliminary experiments. In addition, to evaluate the differences in toxic effects between the structures, the toxicities of the PGC and PLGC oligomers were compared at the levels established by preliminary experiments.

The cell viability and LDH concentration in the supernatant in the 0.5 μM DBT-exposed group were significantly lower than those in the control group. Since a significantly lower cell viability due to DBT at the same level for a macrophage cell line, J774.1, was observed in the previous study[11] the cell viability of astrocytes may be as sensitive to DBT as it is to macrophages. Similar to the immune system, the neurotoxicity of DBT may be shown by the cytotoxic effects of DBT on astrocytes. Mizuhashi *et al.*[18] demonstrated that the exposure to TBT at 1 μM did not affect the cell viability of cultured astrocytes of murine cerebral cortex. TBT at 1 μM for 96 h and 3 μM for 24 h and over decreased the number of astrocytes in the study.

Although the cell line and cultured astrocytes from postnatal rats are different, DBT may be more toxic than TBT on astrocytes.

A significantly higher concentration of glutamate in the cell supernatant was observed in the 0.25-μM DBT-exposed group, suggesting that DBT causes functional impairment of the glutamate receptors in the cells causing the release of glutamate into the supernatant. Even at the level that did not induce cell death, the receptors that uptake into the cell may be impaired by DBT and the release of glutamate may increase. Inhibition of cell proliferation was also observed in the 0.25-μM DBT-exposed group, and the cell viability of the 0.25-μM DBT-exposed group was significantly lower than that of the control in the experiment of the cell proliferation. It is suggested that the threshold of DBT that induced cell death is 0.25 μM or 0.5 μM. Because 0.25 μM DBT increased the concentration of glutamate in the supernatant and inhibited cell proliferation, the evaluation of glutamate in the supernatant and the inhibition of cell proliferation may be useful indices in an evaluation system that uses CRL-2534, if not for other substances then at least for DBT.

OT did not affect cell viability or glutamate concentration in the supernatant at the concentration of 100 μM. However, inhibition of cell proliferation was observed in the group exposed to 100 μM OT, which suggests that OT at 100 μM induces toxic effects on astrocytes. It is also suggested that the inhibition of cell proliferation was a more sensitive index of toxicity than cell viability.

Exposure to PLLA 5000 or PLLA 3000 did not induce significant differences in cell viability or glutamate concentration. However, the exposure to 10 μg/ml S3 caused significant differences in the cell viability and the LDH in the supernatant compared with the control. In addition, a significant decrease in the glutamate concentration in the supernatant was observed in the group exposed to 5 μg/ml S3, which did not show a decrease in the cell viability. The inhibitory effects of S3 on cell proliferation were higher than that of PLLA 5000 or PLLA 3000. The cellular toxicity of S3 may be due to the toxicity of tin at 590 ppm or the interaction between a large amount of tin and PLLA. The decrease in the glutamate in the supernatant suggests the impairment of the mechanism that releases glutamate from the cell. As indicators of cell proliferation, the uptakes of TetraColor ONE were inhibited for cells exposed to PLLA 5000 and PLLA 3000 at 7 d and S3 at 5 d and 7 d after the exposure. Exposure for a longer term may have stronger effects on the cells. The uptakes of TetraColor ONE may be a sensitive index for the longer exposure. Since the uptakes of TetraColor ONE may be affected by the cell conditions, their index should always be considered with the cell viability.

The cell viability of the groups exposed to 50 μg/ml PGC oligomer or PLGC oligomer were not significantly different from those in the respective controls. However, the group exposed to 50 μg/ml PGC oligomer had a significantly higher mean glutamate value compared with the control, and the group exposed to 50 μg/ml of PLGC oligomer had a significantly lower mean value than did the control. The difference in their forms may cause a difference in their effects on the cell function. The decrease may be due to the impairment of the receptors for release, and the increase may be due to that of the receptor for uptake. Evaluation of the cytotoxic effects on astrocytes by measuring the glutamate in the cell supernatant may be more accurate than the evaluation of cell viability alone, although the glutamate levels released by the cells are sometimes not stable.

PLLA, PGC oligomer, and PLGC oligomer have high molecular weights. The low toxicity of these compounds may be due to their high molecular weight and low cell membrane permeability. As long as these compounds maintain a high molecular weight in the cranium, their cytotoxic effects may not be serious.

In this study, DBT caused cell death at concentrations of 0.25 μM and 0.5 μM and alterations in function and inhibition of cell proliferation from 0.25 μM. The molecular weight of DBT dichloride is 303.84; therefore, 0.25 μM would be equal to 0.76×10^{-2} μg/ml. The artificial dura mater product dissolves in the cerebrospinal fluid by hydrolysis after its clinical application. The degradation of dura mater occurs within 3 months after clinical application.[2] The amount of the product used is limited to 2 g, and the concentration of tin in the product is less than 10 ppm. Cerebrospinal fluid is produced at a volume of 500–600 ml/day and replaced.[19] Under the conditions of gradual degradation of dura mater and exchange of cerebrospinal fluid, the effects by DBT on the central nervous system may not be serious. However, the DBT concentration due to the degrada-

tion of the artificial dura mater may be higher where it is applied. Further studies are warranted to clarify this point.

The clinical application of OT and each PLLA would not cause neurotoxicity because of their low toxicities. The OT content is 9 times higher than that in DBT in the artificial dura mater, but the inhibition of proliferation of astrocytes was observed at about 200 times higher concentration than DBT. The artificial dura mater degraded at the rate of 22 mg (2 g/90 days) under the production of 500 ml of cerebrospinal fluid per day. In this study, only a slight decrease in the cell proliferation was observed at 50 μg/ml of PLLA 5000 after 7 d exposure.

In summary, DBT showed neurotoxicity at 0.25 μM and 0.5 μM. However, use of artificial dura mater probably would not induce serious neurotoxicity in actual clinical application because DBT or other components would not reach cytotoxic concentrations in the cerebrospinal fluid. Using cultured astrocytes makes the evaluation of cytotoxic effects in the central nervous system more useful by examining cell proliferation and the glutamate concentration in the supernatant in addition to cell viability.

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