

Trehalose Liposomes Inhibit the Growth of Glioblastoma Cell *In vitro* and *In vivo*

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ABSTRACT

The inhibitory effects of Trehalose Liposomes (TL) composed of α -D-glycopyranosyl- α -D-glucopyranoside monomyristate and L- α -dimyristoylphosphatidylcholine on the growth of human glioblastoma (U-87MG) cells were evaluated. Induction of apoptosis was observed after fusion and accumulation of TL in U-87MG cell membranes. Increased membrane fluidity of U-87MG cells treated with TL was observed. TL caused apoptosis in U-87MG cells through the activation of mitochondria and apoptosis-inducing factor via a caspase-independent pathway. Tumor weights markedly decreased in orthotopic graft mouse models of glioblastoma (U-87MG) after intravenous administration of TL compared with those in the control group.

Keywords: Trehalose liposome; Nanomedicine; Therapeutic effects; Glioblastoma

ABBREVIATIONS

DMPC: L- α -dimyristoylphosphatidylcholine; TL: Trehalose Liposomes composed of DMPC and TreC14; TreC14: α -D-glycopyranosyl- α -D-glucopyranoside monomyristate

INTRODUCTION

Glioblastoma is the most malignant brain tumor. The standard of care is a combination of radiotherapy and chemotherapy with temozolomide after surgical resection [1]. Treatment with bevacizumab during radiotherapy does not improve survival in patients with glioblastoma [2,3].

Trehalose Liposomes (TL) composed of L- α -Dimyristoylphosphatidylcholine (DMPC) and trehalose micelles have been produced using a sonication method [4,5]. TL are effective in inhibiting the growth of tumor cells without including antitumor agents *in vitro* and *in vivo* [6-8]. Induction of apoptosis has been observed in colon and gastric cancer, hepatocarcinoma, leukemia, lung carcinoma, and breast cancer after treatment with TL [4-8]. The anti-invasive effects of TL on the proliferation of lung carcinomas have been reported [9]. However, the inhibitory effects of TL on the growth of glioblastoma cells have not yet been examined. In this study, we investigated the inhibitory effects of TL on the growth of glioblastoma (U-87MG) cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Preparation of TL

TL were prepared using a VS-N300 sonicator (VELVO, Tokyo, Japan) after 30 mol% DMPC (purity>99%; NOF Co. Ltd., Tokyo, Japan) and 70 mol % trehalose C14 (Dojindo Ltd., Japan) were mixed in 5% glucose solution at 45°C and 300 W, and filtered through a 0.20 μ m filter.

Dynamic light scattering measurements

The diameter (d_{hy}) of the TL was measured using an ELS-8000 light scattering spectrometer (Otsuka Electronics, Osaka, Japan) equipped with an He-Ne laser (633 nm) at a scattering angle of 90°. The d_{hy} was calculated using the Stokes-Einstein formula (Equation 1), where κ is the Boltzmann constant, T is the absolute temperature, η is the viscosity, and D is the diffusion coefficient:

$$d_{hy} = \kappa T / 3\pi\eta D \text{ (Equation 1)}$$

Cell culture

The U-87MG human glioblastoma cell line purchased from ATCC (Manassas, VA, USA) were cultured in MEM (Gibco, Thermo Fisher Scientific, Inc. Waltham, MA, USA) containing 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT,

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USA), penicillin (100 unit/ml), and streptomycin (50 µg/ml). The cells were kept in a humidified incubator at 37°C in an atmosphere of 5% CO₂.

Assessment of half-maximal inhibitory concentration (IC₅₀) of TL *in vitro*

The IC₅₀ of TL for U-87MG cells was determined using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H tetrazolium, monosodium salt] assay (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) [10]. U-87MG cells (~5.0 × 10³ cells/ml) were seeded in 96-well plates and cultured in a 5% CO₂ humidified incubator at 37°C for 24 h. Cells were cultured for a further 48 h after the addition of DMPC (0.2.0 × 10⁻³ M) and TL (0.6.0 × 10⁻⁴ M). The WST-8 solution was then added and incubated for 3 h. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific). The inhibitory activity of TL on the growth of U-87MG cells was evaluated by calculating A_{mean}/A_{control}, where A_{mean} and A_{control} denote the absorbance of water-soluble formazan in the presence and absence of TL, respectively.

Analysis of induction of apoptosis by TL

Cells were seeded at a density of 5.0 × 10³ cells/100 ml in a culture dish and incubated in a humidified atmosphere of 5% CO₂ at 37°C for 24 h. TL (0.1 5.0 × 10⁻⁴ M) was added to each dish and the dishes were incubated.

Cells were centrifuged and suspended in PBS (-) containing 1 mg/ml RNase, 0.1% Triton X-100, and 40 µg/ml propidium iodide (Molecular Probes, Eugene, OR, USA) in a dark room. The percentage of apoptotic cells was analyzed using an Epics XL system II flow cytometer (Beckman Coulter, Brea, CA, USA).

Measurement of caspase activity

U-87MG cells (5.0 × 10⁴ cells/ml) were treated with TL. After 24 h of incubation, each substrate solution (PhiPhiLux-GID2, CaspaLux 8-LID2, CaspaLux 9-MID2 for caspase-3, caspase-8, and caspase-9, respectively; OncoImmunin Inc., Gaithersburg, MD, USA) was added to the U-87MG cell suspension [11]. The cells were incubated for 45 min at 37°C, washed twice with 1 ml of ice-cold PBS (-), and resuspended in 1 ml fresh PBS (-). Caspase activity was analyzed by flow cytometry.

Mitochondrial membrane potential

3, 3-Dihexyloxycarbocyanine iodide (DiOC6 (3); Molecular Probes, Eugene, OR, USA) was added to U-87MG cells (5.0 × 10⁴ cells/ml), previously treated with TL (2.5 × 10⁻⁴ M), to evaluate mitochondrial transmembrane potential (Δψ_m). Stained U-87MG cells were examined by flow cytometry using a 15 mW 488 nm air-cooled Ar laser and an FL1 sensor (505-545 nm).

Assessment of Apoptosis Inducing Factor (AIF) by flow cytometry

U-87MG cells (5.0 × 10⁴ cells/ml) were seeded in dishes and incubated for 24 h. The cells were then treated with TL (2.5 × 10⁻⁴ M) during another 24 h incubation. The cells were then fixed with 10% formaldehyde for 30 min in wells, followed by incubation with anti-AIF Alexa Fluor® 488-conjugated antibody (5 µg/ml; Abcam plc, Cambridge, UK) in a shaded box at 4°C for 1 h. The stained U-87MG cells were analyzed by flow cytometry.

Fusion and accumulation of TL in cell membrane

TL fusion and accumulation in the cell membrane was assessed using confocal laser microscopy (TCS-SP; Leica Microsystems, Berlin, Germany) and a fluorescent probe ((1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-glycero-3-phosphocholine (NBDPC; Avanti Polar Lipids, Birmingham, ALUSA)). U-87MG cells (5.0 × 10⁴ cells/ml) were incubated in a humidified incubator at 37°C and 5% CO₂ for 24 h. The cells were then treated with TL/NBDPC ([DMTre]=9.6 × 10⁻⁵ M, [NBDPC]=3.9 × 10⁻⁶ M~4.0 × 10⁻⁶ M) for 10-60 min and examined by flow cytometry using a 15 mW 488 nm air-cooled Ar laser and FL1 sensor (505-545 nm).

Measurement of cellular membrane fluidity

The membrane fluidity of U-87MG cells was measured by spectrophotometry (F-7100; HITACHI, Tokyo, Japan) based on a previously described fluorescence depolarization method [12]. The cells were labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH; Nacalai Tesque, Tokyo, Japan). The fluorescence depolarization (P) value of DPH in cells treated with TL (5.0 × 10⁻⁵ M) was measured.

Assessment of therapeutic effects of TL *in vivo*

All mice were handled in accordance with the guidelines for animal experimentation mandated by Japanese law. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals issued by Sojo University.

The protocol was approved by the Committee on Ethics of Animal Experiments at Sojo University. BALB/c-R/J mice were kindly provided by Prof. Seiji Okada (Kumamoto University, Japan) [13].

The mice were bred using 100% fresh ventilatory changes (14 times per hour) at a room temperature of 25 ± 1°C, humidity of 50 ± 10%, and a light/dark cycle of 12 h.

The mice were randomly grouped based on their body weights using the stratified randomization method. Five mice were included in each group. U-87MG cells (2.5 × 10⁶ cells in 10 µL Matrigel) were orthotopically implanted into the right brains of anesthetized mice.

TL (136 mg/kg for DMPC) was intravenously administered once daily for 29 days starting one day after inoculation with U-87MG cells. Brain samples were harvested from anesthetized mice and weighed after completion of the TL administration regime.

Statistical analysis

Data are presented as mean ± S.D. For statistical comparison, Student's t-test was performed. Differences were considered statistically significant at P<0.05.

RESULTS

Physical properties of TL

The diameter of the TL was measured using dynamic light scattering measurements. The hydrodynamic diameter of TL was <100 nm, indicating that TL can avoid recognition by the reticular endothelial system *in vivo* [14]. The DMPC liposomes were unstable and precipitated.

TL inhibition of U-87MG cell growth leading to apoptosis

The 50% Inhibitory Concentration (IC₅₀) of TL is shown in Figure 1A. The IC₅₀ value was 92 μM, whereas that of the DMPC liposomes was 240 μM. The difference was significant, indicating a greater inhibitory effect of TL compared with that of DMPC liposomes. Induction of apoptosis was investigated using flow cytometry. The amount of apoptotic DNA increased in U-87MG cells treated with TL and reached 100% (Figure 1B).

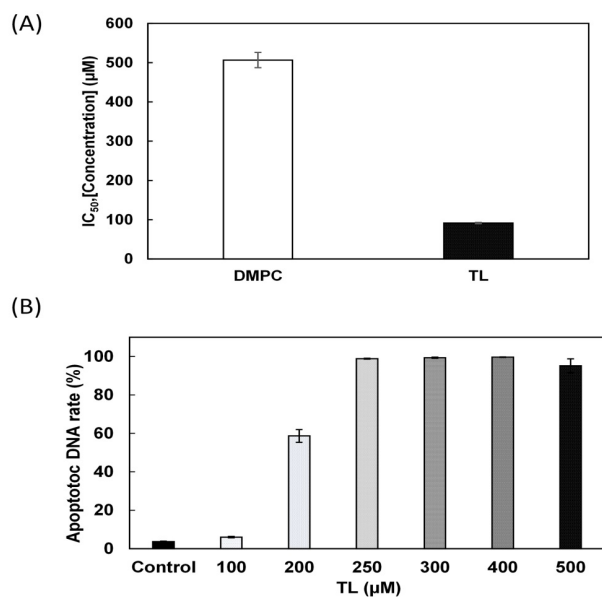


Figure 1: (A) Inhibitory effects of TL on the growth of U-87MG human glioblastoma cells. (B) Apoptotic DNA rate for U-87MG cells treated with TL for 48 h. *P<0.05 (*vs.* control).

Apoptotic signal transduction by TL

The apoptotic pathway in U-87MG cells treated with TL was examined. Caspase-3, -8, and -9 were not activated in U-87MG cells treated with TL, indicating that TL induced apoptosis through a caspase-independent pathway (Figure 2A). The mitochondrial pathway in U-87MG cells treated with TL was also examined. The mitochondrial transmembrane potential of U-87MG cells treated with TL decreased, indicating that TL

induced apoptosis in U-87MG cells through the mitochondria (Figure 2B). AIF is involved in initiating the caspase-independent pathway of apoptosis. Thus, the expression of AIF was assessed. The fluorescence intensity of AIF increased in U-87MG cells treated with TL, indicating that AIF is involved in apoptosis induced by TL (Figure 2C). These results suggest that TL induces apoptosis through mitochondria and AIF in a caspase-independent pathway manner.

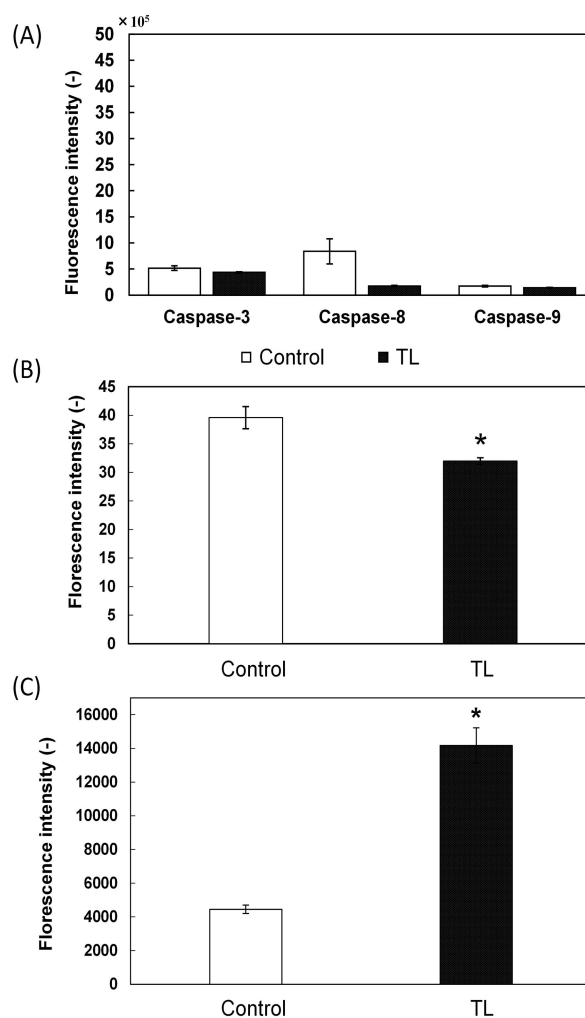


Figure 2: (A) Inactivation of caspase-3, -8, and -9 in U-87MG cells treated with TL. (B) Decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) for U-87MG cells treated with TL. (C) Relative expression rate of AIF in U-87MG cells after treatment with TL for 24 h.

Accumulation of TL in cell membrane

The fusion and accumulation of TL membranes of U-87MG cells was examined using fluorescence probe-labeled TL. The fluorescence intensity of labeled TL increased (Figure 3A), indicating that TL fused and accumulated in the membrane of U-87MG cells.

The membrane fluidity of the U-87MG cells treated with TL was examined. The Pvalue decreased for U-87MG cells treated with TL, indicating that the membrane fluidity of U-87MG cells increased after TL treatment (Figure 3B).

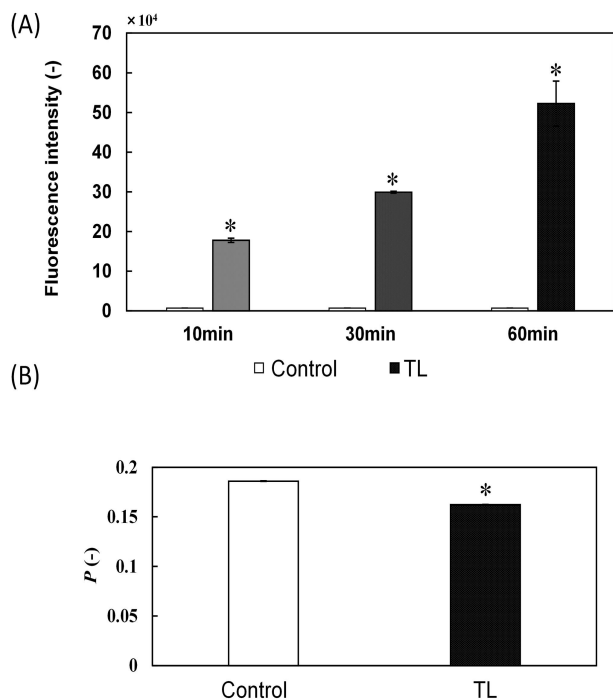


Figure 3: (A) Fusion and accumulation of TL, including NBDPC. (B) Increase in membrane fluidity of U-87MG cells after treatment with TL * P<0.05 (vs. control and DMPC).

Reduction in tumor area in brains of orthotopic graft mouse model of glioblastoma after treatment with TL

We examined the therapeutic effects of intravenous TL treatment on tumor growth in the orthotopic graft mouse model of glioblastoma after the inoculation of U-87MG cells.

Analysis of autopsy photographs of tumors in the mice revealed the reduction in tumor growth (Figure 4A).

Remarkable inhibition of tumor enlargement (vs. control, P<0.05) was observed in the mice treated with TL based on relative brain weight (Figure 4B).

These results suggest that TL is remarkably effective at inhibiting the growth of U-87MG cells and can cross the blood-brain barrier.

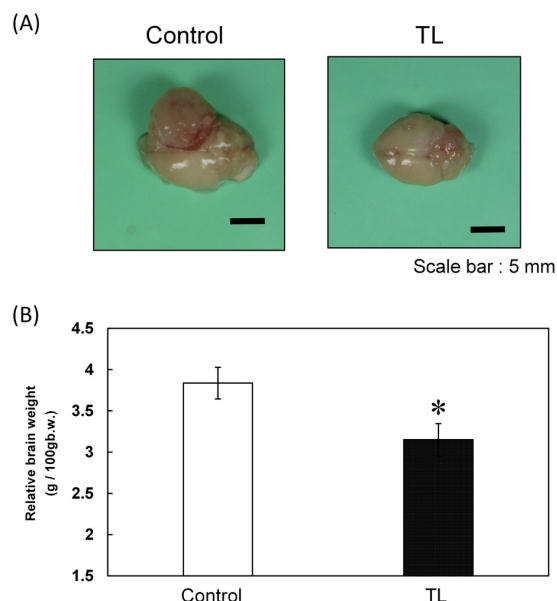


Figure 4: (A) Photographs of brains from orthotopic graft model mice intravenously treated with TL after the inoculation of U-87MG cells. (B) Relative brain weight of orthotopic graft model mice treated with TL after inoculation of U-87MG cells. *P<0.05 (vs. control).

DISCUSSION

The findings are the first evidence of the therapeutic effect of TL in an orthotopic graft mouse model involving U-87MG human glioblastoma cells. There are five noteworthy findings. First, TL with a hydrodynamic diameter <100 nm persisted for 4 weeks. Second, TL inhibited the growth of U-87MG cells. This inhibition was driven by increased apoptosis. Third, TL induced apoptosis in U-87MG cells via the release of AIF in a caspase-independent pathway manner. Fourth, TL fused and accumulated in U-87MG cell membranes, increasing their fluidity. Fifth, TL also inhibited tumor enlargement in an orthotopic graft mouse model of glioblastoma.

CONCLUSION

The findings are the first evidence of the therapeutic effect of TL in an orthotopic graft mouse model involving U-87MG human glioblastoma cells. The results could be potentially advantageous for future clinical applications of TL therapy in patients with glioblastoma.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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