

Active Component of *Fatsia japonica* Enhances the Transduction Efficiency of Tat-SOD Fusion Protein both *In Vitro* and *In Vivo*

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It has been reported that Tat-SOD can be directly transduced into mammalian cells and skin and acts as a potential therapeutic protein in various diseases. To isolate the compound that can enhance the transduction efficiency of Tat-SOD, we screened a number of natural products. 3-O- $[\beta$ -D-Glucopyranosyl(1 \rightarrow 4)- α -L-arabinopyranosyl]-hederagenin (OGAH) was identified as an active component of *Fatsia japonica* and is known as triterpenoid glycosides (hederagenin saponins). OGAH enhanced the transduction efficiencies of Tat-SOD into HeLa cells and mice skin. The enzymatic activities in the presence of OGAH were markedly increased *in vitro* and *in vivo* when compared with the controls. Although the mechanism is not fully understood, we suggest that OGAH, the active component of *Fatsia japonica*, might change the conformation of the membrane structure and it may be useful as an ingredient in anti-aging cosmetics or as a stimulator of therapeutic proteins that can be used in various disorders related to reactive oxygen species (ROS).

Keywords: *Fatsia japonica*, Tat-SOD, protein therapy, protein transduction efficiency

Reactive oxygen species (ROS) are inevitably formed as by-products of various normal cellular processes involving interactions with oxygen. These ROS damage macromolecules, which are constantly exposed to the harmful actions of ROS. Ultimately, these ROS contribute significantly to the pathological processes of various human diseases, which include ischemia, carcinogenesis, radiation injury, and

inflammation/immune injury [13, 15]. Cellular defense against ROS utilizes antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase [29]. Cu,Zn-SOD is thought to provide a primary line of defense by catalyzing the dismutation of ROS [2].

Since many biological macromolecules can be damaged by oxygen radicals, interest has grown in the therapeutic potential of SOD [10, 32]. Gene therapy is considered a promising method for introducing therapeutic proteins into cells, but this approach is somewhat constrained by problems of gene delivery, delayed gene expression, inefficiency, and toxicity [38].

Recently, several small regions of proteins, called protein transduction domains (PTDs), have been developed to allow the delivery of exogenous protein into living cells. To date many researchers have demonstrated the successful delivery of full-length Tat fusion proteins by protein transduction technology [8]. We successfully transduced Tat-SOD directly into insulin-producing RINm-5F and pancreatic islet cells and transduced Tat-SOD by increasing the radical scavenger activity in the pancreas [11]. Recently, we showed that the Tat-pyridoxal kinase (Tat-PK) and Tat-pyridoxal oxidase (Tat-PO) fusion proteins were efficiently transduced into PC12 cells and catalytically active in the cells [23, 25]. Moreover, we transduced PEP-1-SOD into neuronal cells and across the blood-brain barrier, which efficiently protected against ischemic insults, and the Tat- α -synuclein fusion protein protects against oxidative stress *in vitro* and *in vivo* [6, 12].

Fatsia japonica, belonging to the family Araliaceae, is a ubiquitous evergreen plant found in both Japan and Korea. It is an erect shrub with few branches and can grow to a height of 2.5 m. Its large leaves are rich and glossy in color [34]. All members of plants in the Araliaceae family, such as *Hedera helix*, *Brassaia actinophylla*, *Schefflera arboricola*,

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and *Dendropanax trifidus*, are reported to cause contact dermatitis, and the major allergen has been demonstrated to be faltarinol [14, 16, 17, 35]. However, faltarinol was reported to be absent in *Fatsia japonica* [4].

In view of the known biological actions of *Fatsia japonica* on the cell membrane and skin, we investigated whether it could enhance the transduction of the cell-permeable antioxidant enzyme Tat-SOD *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Cell-permeable Tat-SOD was expressed in *E. coli* and purified as described previously [27]. Ni²⁺-nitrilotriacetic acid Sepharose Superflow was purchased from Qiagen (Valencia, CA, U.S.A.). Isopropyl- β -D-thiogalactoside (IPTG) was obtained from Duchefa Co. (Haarlem, The Netherlands). Rabbit anti-histidine polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and all other chemicals and reagents were the highest analytical grade available. The stems of *Fatsia japonica* Dence et Planch (Araliaceae) were collected in Seogwipo, Jeju Province, Korea, in June 2004. A voucher specimen was deposited in the Herbarium of the Regional Innovation Center, at Hallym University, Chunchon, Korea.

General Experimental Procedures

Mp was measured with a Fisher-Johns melting-point apparatus without correction. Optical rotation was determined with a Rudolph Research Autopol III polarimeter. ¹H- and ¹³C-NMR spectra were obtained on a Bruker AC-300 spectrometer with tetramethylsilane (TMS) as internal standard.

Isolation of Active Compound from *Fatsia japonica*

The dried stems of *Fatsia japonica* (1.0 kg) were extracted with 95% EtOH. After removal of the solvent by evaporation, the residue (67.3 g) was suspended in H₂O (1.2 liter). Successive fractionation with *n*-hexane, chloroform, ethylacetate, and water-residue yielded four fractions: an *n*-hexane-soluble fraction (4.3 g), a chloroform-soluble fraction (6.9 g), an ethylacetate-soluble fraction (12.9 g), a water-residue fraction (40.2 g). The water-residue fraction (40.0 g) was chromatographed over Diaion HP-20 with stepwise elution of H₂O, 25% MeOH, 40% MeOH, 65% MeOH, 80% MeOH, and MeOH furnishing six fractions: fraction 1 (7.9 g), fraction 2 (3.2 g), fraction 3 (5.9 g), fraction 4 (6.9 g), fraction 5 (3.1 g), and fraction 6 (10.5 g). Fraction 5 was subsequently subjected to ODS column chromatography (JAI, recycle HPLC system, Japan). Elution with 30% MeOH yielded active components (36.4 mg). The structure of the active component was assigned as 3-O- $[\beta$ -D-glucopyranosyl (1 \rightarrow 4)- α -L-arabinopyranosyl]-hederagenin (OGAH) by chemical and spectral examinations.

OGAH

Colorless needles (MeOH); mp 246-248°; $[\alpha]^{16}_D +44.6^\circ$ (c=0.79, pyridine); ¹H NMR (pyridine-*d*₅) δ 0.84, 0.95, 0.99, 1.00, 1.04, 1.23 (each 3H, s, *tert*-Me), 3.30 (1H, dd, *J*=4.0 and 13.5 Hz, H-18), 3.65-3.80 (3H, m, H-3 and sugar-H), 4.03-4.30 (6H, m, H₂-23 and sugar-H), 4.36 (1H-dd, *J*=4.0 and 12.0 Hz glycosyl H-6), 4.45 (1H, dd, *J*=2.5 and 12.0 Hz, glycosyl H-6), 4.56 (1H, t, *J*=6.5 Hz,

arabinosyl H-2), 5.20 (1H, d, *J*=6.5 Hz, arabinosyl H-1), 5.21 (1H, d, *J*=7.5 Hz, glycosyl H-1), 5.49 (1H, brs, H-12); ¹³C NMR (pyridine-*d*₅) δ 13.5 (C-24), 16.1 (C-25), 7.5 (C-26), 18.3 (C-6), 23.7(C-11), 23.8 (C-30), 23.9 (C-16), 26.0 (C-2), 28.4 (C-15), 31.0 (c-20), 33.0 (C-7), 33.3 (C-22), 33.3 (C-29), 34.3 (C-21), 37.0 (C-10), 38.8 (C-1), 39.8 (C-18), 42.0 (C-18), 42.2 (C-14), 43.6 (C-4), 46.5 (C-19), 46.7 (C-17), 48.0 (C-5), 48.2 (C-9), 62.6 (glycosyl C-6), 64.9 (C-23), 65.0 (arabinosyl C-5), 68.3 (arabinosyl C-3), 71.5 (glycosyl C-4), 73.7 (arabinosyl C-2), 76.3 (glycosyl C-2), 78.3 (glycosyl C-5), 81.5 (arabinosyl C-4), 82.3 (C-3), 104.0 (arabinosyl C-1), 105.0 (glycosyl C-1), 122.6 (C12), 144.9 (C-13), 180.2 (C-28).

Acid Hydrolysis of OGAH

A solution of active components (10 mg) in 2% H₂SO₄ (2 ml) was hydrolyzed. The reaction mixture was worked up and yielded hederagenin (3 mg). Cellulose in the examination of the aqueous layer showed the presence of arabinose and glucose.

Effects of OGAH on Tat-SOD Activity and Transduction

The HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 20 mM HEPES/NaOH (pH 7.4), 5 mM NaHCO₃, 10% fetal bovine serum, and antibiotics (100 μ g/ml streptomycin, 100 U/ml penicillin) at 37°C under a humidified condition of 95% air and 5% CO₂.

OGAH was isolated from *Fatsia japonica*. OGAH, at a concentration of 10 mg/ml, was dried and stored at -20°C. To assess the transduction and enzyme activity, the samples were diluted with deionized distilled water. Samples were pretreated with OGAH (50 μ g/ml) for 12 h, and exposed to various concentrations of Tat-SOD for 1 h. They were then harvested and cell extracts used for enzyme assays and Western blot analyses.

Western Blot Analysis

The transduced Tat-SOD fusion proteins on the polyacrylamide gel were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% Tween-20 (TBST) for 2 h and was then incubated for 1 h at room temperature with anti-histidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.; dilution 1:400) in TBST. After washing, the membrane was incubated for 1 h with a proper secondary antibody conjugated to horseradish peroxidase diluted 1:10,000 in TBST. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL.

SOD Activity Assay

The SOD activity was measured by monitoring the inhibition of ferricytochrome *c* reduction by the xanthine/xanthine oxidase reaction described by McCord and Fridovich [30]. One unit of enzyme activity was defined as the amount of SOD required to inhibit the rate of reduction of cytochrome *c* by 50%. The protein concentration was determined by the Bradford method using bovine serum albumin as the standard [5].

Measurement of OGAH Cytotoxicity

The cytotoxicity of OGAH on the growth of HeLa cells was assessed using the MTT assay [20, 28]. The cells were seeded in 24-well plates at 70% confluence and cultured for 6 h. Then, the cells were treated with the OGAH (10-50 μ g/ml) for 12 h. After the

fresh culture medium was replaced, the cells were incubated for a further 12 h. MTT solution was added, and the plates were incubated for 4 h. Subsequently, 100 μ l of the supernatant was added to 100 μ l of DMSO. Absorbance of each well was read at 570 nm with a micro ELISA reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The percent of cell survival was defined as the relative absorbance of treated versus untreated cells.

Immunohistochemistry

Mice were housed at a constant temperature (23°C) and relative humidity (60%) with a fixed 12 h light/dark cycle and free access to food and water. After animals had been anesthetized with 3% isoflurane in nitrogen and oxygen, 50 μ g of control SOD, Tat-SOD, and OGAH (100 μ g/ml) combined with Tat-SOD were topically applied onto shaved areas of mouse skin for 1 h. Thereafter, frozen and sectioned tissues were prepared and fixed with 4% paraformaldehyde for 10 min. To remove nonspecific immunoreactivity, free-floating sections were first incubated with 0.3% Triton X-100 and 10% normal goat serum in PBS for 1 h at room temperature. They were then incubated with rabbit anti-histidine IgG (1:500) for 24 h at room temperature. After washing three times for 10 min with PBS, the sections were incubated for 1 h with biotinylated goat anti-rabbit IgG (Vector Laboratories, U.S.A.; dilution 1:200), and then visualized with 3,3'-diaminobenzidine (40 mg DAB/0.045% H₂O₂ in 100 ml of PBS) and mounted on gelatin-coated slides. Immunoreactions were observed with an Axioscope microscope (Carl Zeiss, Germany).

RESULTS

Effects of *Fatsia japonica* Crude Extract on Tat-SOD Transduction

We have already reported that the Tat-SOD fusion proteins are transduced into HeLa cells, and have a protective effect against oxidative stress [27]. In the present study, we investigated the effects of various natural products (> 20) on the transduction of Tat-SOD into mammalian cells. Among the various natural products, crude extracts of *Fatsia japonica* markedly increased Tat-SOD transduction efficiency and enzymatic activities. In the crude extract of *Fatsia japonica* (1–2 mg/ml) treated group, the transduction efficiency of Tat-SOD and SOD activities was about two times higher than in the Tat-SOD control group (Fig. 1). Moreover, crude extract of *Fatsia japonica* had no effect on the cell cytotoxicity (data not shown).

Isolation of Active Compound from *Fatsia japonica*

The procedure used to fractionate the active extract from *Fatsia japonica* stems is described in Methods and Materials. The active fractions, water-soluble fraction and fraction 5, were subjected to Diaion HP-20 and ODS column chromatography. The eluents were then analyzed for transduction efficiency. The ¹H-NMR and ¹³C-NMR spectra showed that the active components of *Fatsia japonica* were 3-O-[β -D-glucopyranosyl(1 \rightarrow 4)- α -L-arabinopyranosyl]-hederagenin (OGAH). Finally, 36.4 mg of components was obtained from 1 kg of stems of *Fatsia japonica* (Fig. 2).

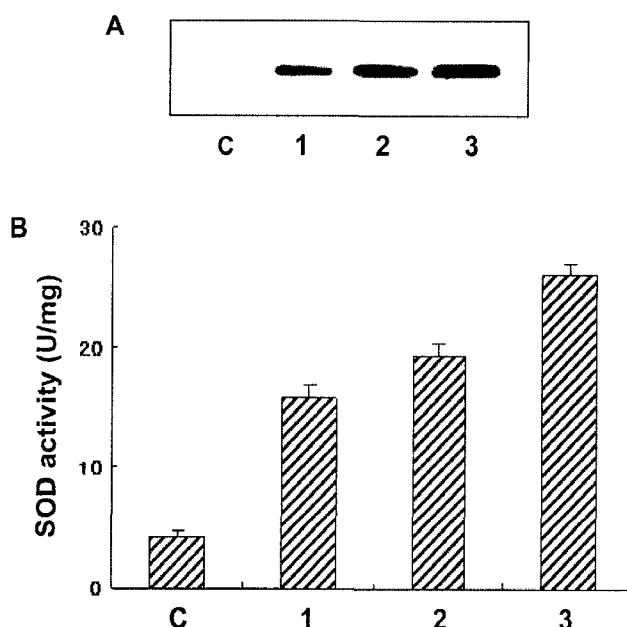


Fig. 1. Tat-SOD transduction into HeLa cells by *Fatsia japonica* crude extracts.

HeLa cells were pretreated with crude extracts (1–2 mg/ml) for 12 h. Then, Tat-SOD (3 μ M) was added to the culture medium for 1 h. Transduced Tat-SOD and enzymatic activities were analyzed by Western blotting and SOD activity. **A** and **B** are as follows: lane 1, Tat-SOD treated cells; lane 2, Tat-SOD and crude extract (1 mg/ml) treated cells; lane 3, Tat-SOD and crude extract (2 mg/ml) treated cells.

Effects of OGAH on Tat-SOD Transduction

To investigate the effect of OGAH on the transduction of Tat-SOD fusion protein into mammalian cells, we performed a Western blot using anti-histidine antibody and enzyme assays. Various concentrations of Tat-SOD (1–3 μ M) fusion proteins were added to a culture media of HeLa cells for 1 h. Additionally, Tat-SOD fusion proteins were added to the culture media of HeLa cells at 3 μ M concentration for various times (10–60 min). As shown in Fig. 3, the levels of transduced Tat-SOD fusion proteins in the cultured HeLa cells significantly increased in a dose- and time-dependent manner when the cells were preincubated with OGAH for 12 h.

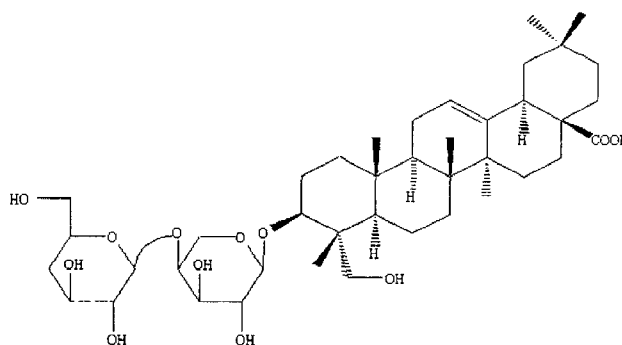


Fig. 2. The structure of 3-O-[β -D-glucopyranosyl(1 \rightarrow 4)- α -L-arabinopyranosyl]-hederagenin (OGAH).

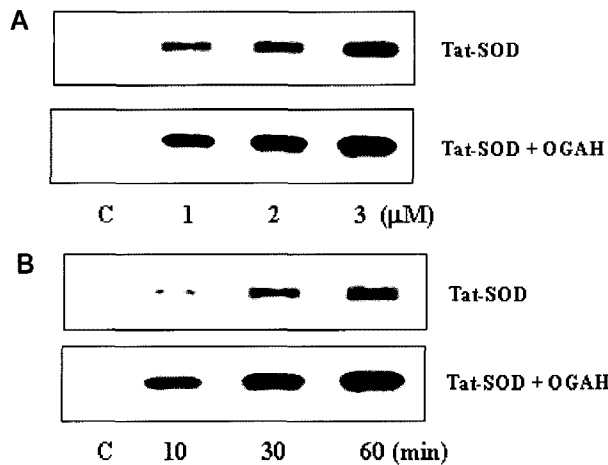


Fig. 3. Dose- and time-dependent transduction of Tat-SOD into cultured HeLa cells. 1–3 μM of Tat-SOD was added to the culture medium for 1 h (A), 3 μM of Tat-SOD was added to the culture medium for 10–60 min (B). Cells were pretreated with OGAH (50 $\mu\text{g}/\text{ml}$) for 12 h. Transduced Tat-SOD was analyzed by Western blotting.

Expression of the enzymatic activity of the transduced Tat-SOD fusion proteins is essential for any therapeutic application. Therefore, we determined the dismutation activities of SOD in HeLa cells treated with Tat-SOD and OGAH. As shown in Fig. 4, SOD activity markedly

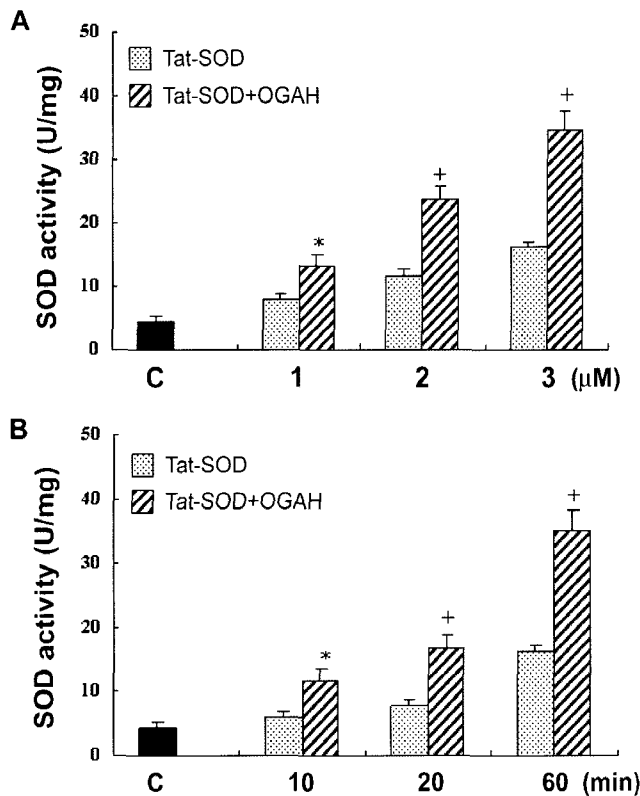


Fig. 4. The specific activities of SOD in transduced HeLa cells. Cells were treated as in the legend to Fig. 3. Each bar represents the mean \pm SEM. of four experiments. Asterisks ($P < 0.05$) and crosses ($P < 0.01$) denote values that are significantly different from that of Tat-SOD on its own. Significance was evaluated by a Student's *t*-test.

increased in a dose- and time-dependent manner in the cells that had been treated with the OGAH.

Effect of OGAH on the Cell Toxicity and Tat-SOD Transduction Efficiency

To evaluate the effects of OGAH on Tat-SOD transduction efficiency to the cells, the HeLa cells were preincubated with OGAH (10–50 $\mu\text{g}/\text{ml}$) for 12 h. As shown in Fig. 5A, the levels of transduced Tat-SOD fusion proteins in the cells increased in an OGAH dose-dependent manner.

The effect of OGAH on the cell toxicity was investigated with the MTT assay. After the cells were seeded 24-well plates, various concentrations of OGAH (10–50 $\mu\text{g}/\text{ml}$) were added. As shown in Fig. 5B, we could not observe any significant change of cell viability after 12 h of treatment with OGAH in HeLa cells. These results indicate that OGAH did not have any cytotoxic effect on cell growth, whereas it elevated the transduction efficiency of Tat-SOD fusion protein.

Effect of OGAH on Transduction of Tat-SOD into Mice Skin

We examined the effect of OGAH on the transduction of Tat-SOD fusion protein into mice skin by immunohistochemistry and by determining SOD activity. As shown in Fig. 5, transduction efficiency and SOD enzyme activity were markedly increased by the presence of OGAH. The transduction signals were significantly detected in the epidermis and dermis of skins. In addition, the levels of enzyme activities in skin increased approximately five to six times compared with the control. These results demonstrated that the active components of *Fatsia japonica*, OGAH,

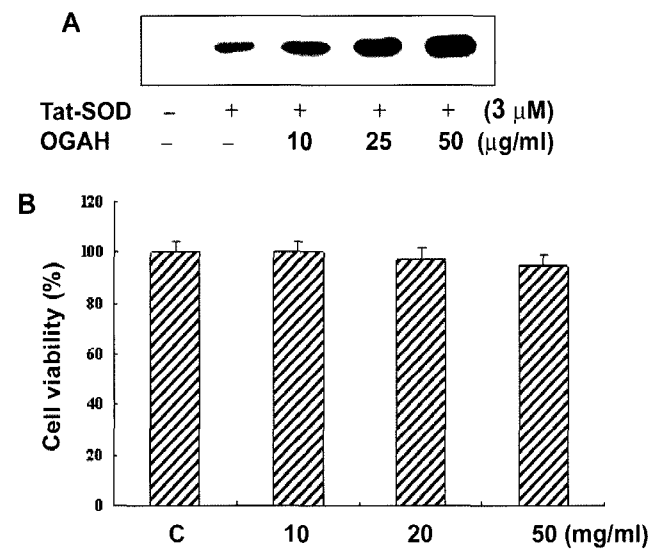


Fig. 5. Effect of OGAH on cell viability and Tat-SOD transduction efficiency. Cells were incubated with OGAH (10–50 $\mu\text{g}/\text{ml}$) for 12 h. Transduced Tat-SOD was analyzed by Western blotting (A) and cell viabilities were estimated by a colorimetric assay using MTT (B).

enhance the transduction efficiency of Tat-SOD fusion protein *in vitro* and *in vivo*.

DISCUSSION

It is well known that Cu,Zn-SOD is one of the cell's primary defenses against oxygen-derived free radicals, and is vital for maintaining a healthy balance between oxidants and antioxidants. To develop a system for overexpressing SOD and to purify it, we constructed Tat-SOD expression vectors, containing in tandem the cDNA sequences for human Cu,Zn-SOD, and Tat-protein (Tat 49-57), transduction domain, and six histidine residues at the amino-terminus. We have already reported that the Tat-SOD fusion proteins are transduced into HeLa cells and have a protective effect against oxidative stress [27].

Fatsia japonica (Araliaceae) (Korean name: Phalsonii) is commonly cultivated in gardens for their handsome foliage. It is also used in various folk remedies [34]. A number of triterpenoid saponins have been isolated from the flowers, mature fruits, and leaves of this species [8, 26]. However, its precise mechanism for changing the conformation of cell membrane and skin tissue are unknown. In the present study, we investigated the effects of the active components of *Fatsia japonica*, 3-O-[β -D-Glucopyranosyl (1 \rightarrow 4)- α -L-arabinopyranosyl]-hederagenin (OGAH), on the transduction of Tat-SOD into mammalian cells and animal skins.

Tat-SOD transduction efficiency and enzyme activities were significantly increased by the presence of the crude extract of *Fatsia japonica*. The *Fatsia japonica* may modify the cellular membrane structure, leading to increased membrane fluidity. The cell membrane may then be more flexible and unstable, permitting increased protein transduction.

During the isolation of the components in the *Fatsia japonica*, we observed the highest transduction efficiency in the water-residue fraction. This fraction was further analyzed by Diaion HP-20 and ODS column chromatography. The structure of the active components from *Fatsia japonica* was identified as OGAH (Fig. 2). As shown in Figs. 3 and 4, the transduction efficiency and enzyme activities were significantly increased when treated with OGAH. However, OGAH did not have any cytotoxic effect on the cell (Fig. 5). All plants of the raliaceae, such as *Hedera helix* L., *Hedera canariensis*, and *Schefflera arboricola*, are reported to cause contact dermatitis [14, 31, 35]. Falcarinol has been found to act as a major allergen of the plant and was first isolated from *Panax ginseng* C. A. Mayer [14, 37]. In the present study, this active component of *Fatsia japonica* was found to be a triterpenoid glycoside. Saponins are triterpenoid glycosides are widespread in terrestrial and marine plants and possess various biological and pharmacological activities. Moreover, hederagenin saponins are largely represented in

nature and possess many biological activities such as hemolytic, antiviral, antimicrobial, fungicidal, molluscicidal, and cytotoxic effects [19]. Although many reports have shown that saponins affect membrane proteins and membrane channels [3, 24], its precise mechanism of action is unknown. As a negative control, we examined the effect of SOD activity and cellular expression levels by OGAH. The OGAH did not show any effect on SOD activity in *in vitro* experiment, and when the OGAH was treated to the HeLa cells, the OGAH itself did not affect any SOD activities and expression levels. However, the OGAH increased the transduction of Tat-SOD into the cells as a function of OGAH concentration. Therefore, as shown in Fig. 5, the Tat-SOD transduced into cells as a function of OGAH concentrations (Fig. 5A) and the cell viability does not change at all (Fig. 5B). These results indicate that the OGAH does not have any cytotoxicity to the cells.

We also examined the effect of OGAH on the penetration of Tat-SOD fusion protein into mice skin by immunohistochemistry and by determining SOD activity. As shown in Fig. 6B, transduction efficiency was significantly increased by the presence of OGAH. We have already suggested that the transduction of SOD, catalase, and ribosomal protein S3 (RPS3) could be used in anti-aging cosmetics or in therapy for various disorders that are related to reactive oxygen species [20, 22, 27]. However, the use of saponins

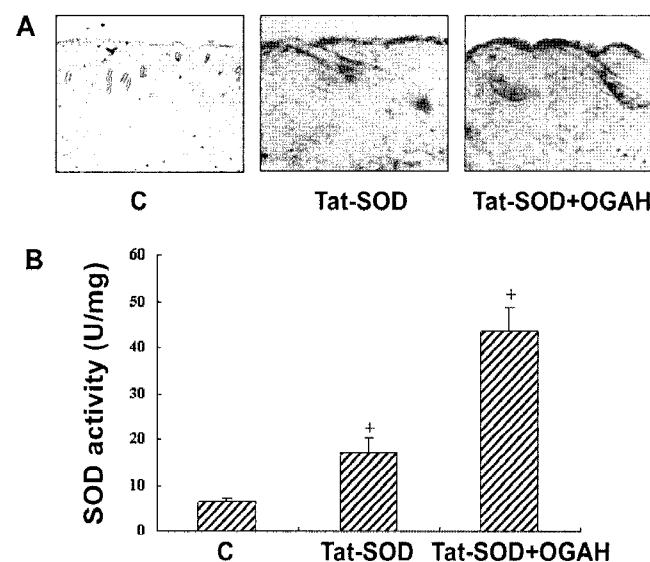


Fig. 6. Immunohistochemical analysis of animal skin transduced with Tat-SOD proteins.

Tat-SOD (50 μ g) was applied topically onto a shaved area of the mice's dorsal skin for 1 h. Frozen sections of the skin tissues were immunostained with a rabbit anti-histidine IgG, as described in Materials and Methods. The sections were visualized with 3,3'-diaminobenzidine and observed with an Axioscope microscope (A). Transduction efficiencies were analyzed by measuring the specific enzyme activities of the skin tissue (B). Crosses ($P < 0.01$) denote values that are significantly different from the control and Tat-SOD, respectively, as evaluated by Student's *t*-test. Each bar represents the mean \pm SEM. of three experiments.

in skin-related disorders has been relatively limited. In the 1980's, the anti-inflammatory effects of ginseng saponin ointment [22] and the effects of ginseng powder and ginseng saponin on *Candida albicans* [33] were reported. In the cosmetic field, ginseng extract in a liposome preparation had a restorative effect on sagging and wrinkled skin [36] as well as on aging skin, cutaneous elasticity, desiccation of the stratum corneum of the epidermis, and circulatory derangements [9]. Estrone, estradiol, and estriol were isolated from *Panax ginseng* root as cutaneous bioactive components [1] and the use of topical ginseng appears to have an estrogen-like effect on genital tissues and helps reduce postmenopausal bleeding [18].

In summary, the results of this study show that OGAH, which are known active components of *Fatsia japonica*, enhances the transduction of cell-permeable Tat-SOD *in vitro* and *in vivo*. Accordingly, it may be useful as a cofactor in anti-aging cosmetics and in protein therapy for various disorders related to the antioxidant enzymes Cu,Zn-SOD or reactive oxygen species.

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