Enhancement of HIV-1 Tat fusion protein transduction efficiency by bog blueberry anthocyanins

Sun Hwa Lee1,*, Hoon Jae Jeong1,*, Dae Won Kim1,*, Eun Jeong Sohn1, Mi Jin Kim1, Duk Soo Kim2, Tae Cheon Kang3, Soon Sung Lim4, Il Jun Kang4, Sung-Woo Cho5, Kil Soo Lee1, Jinseu Park1, Won Sik Eum1,* & Soo Young Choi1,*

1Department of Biomedical Science & Research Institute of Bioscience and Biotechnology, Hallym University, Chunchon 200-702, 2Department of Anatomy, College of Medicine, Soonchunhyang University, Cheonan 330-090, 3Department of Anatomy & Neurobiology, College of Medicine, Hallym University, Chunchon 200-702, 4Department of Food Science and Nutrition & RIC Center, Hallym University, Chunchon 200-702, 5Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul 138-736, Korea

INTRODUCTION

Though protein transduction domains (PTDs) are well known for the delivery of exogenous therapeutic proteins into living cells, the overall low efficiency of transduction is a serious obstacle. We investigated the effect of bog blueberry anthocyanins (BBA) on protein transduction efficiency and found that BBA enhanced the transduction efficiencies of Tat-SOD fusion protein into HeLa cells and mice skin. The enzymatic activities in the cells and skin tissue in the presence of BBA were markedly increased compared to controls. Further, BBA did not demonstrate any cell toxicity at various concentrations. Although the mechanism is not fully understood, we suggest that BBA might alter the conformation of the membrane, which would indicate that BBA can be used as a protein transduction enhancer for the efficient delivery of therapeutic proteins for a variety of disorders. [BMB reports 2010; 43(8): 561-566]
the effects of the anthocyanins of bog blueberry (BBA) on the transduction of Tat-SOD into HeLa cells and animal skin. As shown in Fig. 1, BBA was identified as cyanidine-3-glucoside, petunidin-3-glucoside, malvidine-3-glucoside, delphinidin-3-glucoside and delphinidine-3-arabinoside.

To determine the cytotoxic effects of BBA on HeLa cells, the cells were treated with various (100-1000 ng/ml) concentrations of BBA and incubated for 12 h. As shown in Fig. 2, BBA did not affect cell viability at various concentrations. These results indicate that BBA was not cytotoxic. Other studies have shown that cyanidine-3-glucoside, petunidin-3-glucoside, malvidine-3-glucoside and delphinidin-3-glucoside are present in red wine where they act as natural colorants. In addition, human consumption of blueberry juice had no negative effects (35-37).

Endogenous SOD activity was not significantly changed when cells were treated with BBA for 12 h (data not shown).

**Effects of BBA on Tat-SOD transduction into cells and skin**

We reported that Tat-SOD fusion proteins are transduced into HeLa cells, where they have a protective effect against oxidative stress (38). In recent decades, protein transduction technologies have been developed for therapeutic purposes (2). In addition, these technologies have been used to successfully transduce a number of different therapeutic proteins both in vitro and in vivo (4). However, protein transduction technology has problems related to transduction efficiency, which make it inadequate for therapeutic applications. Therefore, increasing the transduction efficiency is a very important obstacle that must be overcome for the development of protein therapy technology.

In the present study, we investigated the effects of BBA on the transduction of Tat-SOD into mammalian cells via Western blotting using anti-histidine antibody and enzyme assays. Various concentrations of Tat-SOD (0.5-3 μM) fusion proteins were added to a culture media of HeLa cells for 1 h. Further, Tat-SOD fusion proteins were added to the culture media of HeLa cells at a concentration of 3 μM for various times (10-60 min). As shown in Fig. 3A, the levels of transduced Tat-SOD fusion proteins in cultured HeLa cells significantly increased in a dose- and time-dependent manner when the cells were pre-incubated with BBA for 12 h.

The enzymatic activities of the transduced Tat-SOD fusion proteins must be maintained if any therapeutic application is desired. Therefore, we determined the dismutase activities of SOD in HeLa cells treated with Tat-SOD and BBA. As shown in Fig. 3B, SOD activity markedly increased in a dose- and time-dependent manner in cells treated with BBA. These results indicate that BBA did not impact cell growth and increased Tat-SOD fusion protein transduction efficiency.

As positive control experiments, we examined whether or not BBA increased the transduction efficiency of a control protein such as Tat-GFP. As shown in Fig. 3C, BBA also enhanced the transduction efficiency of Tat-GFP.

Next, we examined the effect of BBA on the transduction of Tat-SOD fusion protein into mice skin by immunohistochemistry and SOD activity. As shown in Fig. 4, the transduction efficiency and SOD enzyme activity were markedly increased by the presence of BBA. In addition, the levels of enzyme activities in skin increased approximately 3-4 fold compared to that treated solely with Tat-SOD. These results demonstrate that BBA enhanced the transduction efficiency of Tat-SOD fusion protein into cells and skin tissue.

Recent studies have demonstrated various methods to enhance transduction efficiency (20, 21). Wang et al. (2010) de-
Enhancement of transduction efficiency by BBA
Sun Hwa Lee, et al.

Fig. 4. Immunohistochemical analysis of animal skin transduced with Tat-SOD proteins. Tat-SOD (50 μg) was applied topically onto a shaved area of mouse dorsal skin for 1 h. Frozen sections of the skin tissues were immunostained with rabbit anti-histidine IgG, as described in Materials and Methods. The sections were visualized with 3,3’-diaminobenzidine and observed using an Axioscope microscope (A). Transduction efficiencies were analyzed by measuring the specific enzyme activities of the skin tissue (B). *P < 0.01 compared with treated with Tat-SOD.

Fig. 3. Effect of Tat-SOD transduction (A), enzyme activity (B) and Tat-GFP (C) in HeLa cells. Dose (0.5-3 μM) and time (10-60 min)-dependent transduction of Tat-fusion protein into cultured HeLa cells. Cells were pretreated with BBA (1,000 ng/ml) for 12 h. Transduced Tat fusion proteins and enzyme activity were analyzed by Western blotting and by measuring specific enzyme activities. *P < 0.01 compared with treated with Tat-SOD.

Monstrated that lower concentrations of DMSO markedly improve Tat fusion protein transduction into cells without cytotoxic effects or perforation of the membrane. In addition, they suggested that application of DMSO as a transduction enhancer is a viable strategy for increasing transduction efficiency. However, high concentrations of DMSO have a repressive effect on cell growth and cytotoxicity, depending on the cell lines in question. Lim et al. (2010) demonstrated that therapeutic protein genes fused with different protein transduction domains (PTDs) such as HIV-1 Tat and 11-arginin tend to overcome low transduction efficiency. Although transduction proteins show protective effects in cells, the protective effects afforded by PTDs are different. In this study, we used a natural product as a transduction efficiency enhancer. Anthocyanins from edible bog blueberries (BBA) have various biological effects on human and animal diseases. We have shown that BBA is non-toxic to cells suggesting it may be used as a protein transduction enhancer without any side effects related to toxicity.

In summary, we demonstrated that BBA enhanced the transduction of Tat-SOD into cell and skin tissue, which supports BBA as an efficient strategy for the delivery of therapeutic proteins. However, the detailed mechanism by which BBA affects transduction requires further study.
MATERIALS AND METHODS

Materials
ICR mice (6-8 weeks) were purchased from the Experimental Animal Center, Hallym University, Chunchon, Korea. The animals used in this experiment were treated according to the “Principles of Laboratory Animal Care” (NIH Publication No. 86-23) approved by the Hallym Medical Center Institutional Animal Care and Use Committee. Cell permeable Tat-SOD was expressed and purified as described previously (38). Rabbit anti-histidine polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and all other chemicals and reagents were of the highest analytical grade available. Edible bog blueberry (Vaccinium uliginosum L., Korean name “Deol-Jugk”) was collected from BeagDu Mountain, North Korea, in August 2005. A voucher specimen was deposited in the Herbarium of the Regional Innovation Mountain, North Korea.

Extraction and identification of bog blueberry anthocyanins
Bog blueberry anthocyanins (BBA) were extracted and identified as previously described (39). Fresh bog blueberries (100 g) were juiced in 100 ml of water, followed by filtration using Whatman No.2 filter paper. After the filtrate was adsorbed on a Dianion HP-20 resin column and washed with water and ethanol, the anthocyanin-rich fractions were obtained. Anthocyanin was flowed through a RP YMC ODS H-80 column using a Finnigan Surveyor HPLC system (ThermoQuest, San Jose, CA) and a Finnigan LCQ Advantage IT mass spectrometer (ThermoQuest, San Jose, CA). The final compounds were confirmed using an m/z value following MS/MS of the m/z value. Five grams of anthocyanins was obtained from 100 g of fresh bog blueberry.

Effects of BBA on Tat-SOD activity and transduction
To measure Tat-SOD transduction and SOD activity, the BBA samples were dissolved in DMSO and then added to HeLa cells. HeLa cells were maintained as described previously (38). Samples were pretreated with BBA (1,000 ng/ml) for 24 h and then exposed to various concentrations of Tat-SOD for 1 h. Cells were then harvested, and the cell extracts were used for enzyme assays and Western blot analyses. SOD activity was measured by monitoring the inhibition of ferricytochrome c reduction by xanthine/xanthine oxidase reaction (40). The protein concentration was determined by the Bradford method using bovine serum albumin as a standard (41).

Western blot analysis
Sample proteins were electrophoretically transferred to a nitrocellulose membrane, after which the membrane was blocked in 5% nonfat milk in TBST buffer (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5 containing 0.05% Tween-20) for 2 h. The membrane was incubated for 1 h at room temperature with anti-histidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 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 bands were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (ECL; Amersham).

Measurement of BBA cytotoxicity
The cytotoxicity of BBA against HeLa cells was measured by MTT assay. The cells were treated with BBA (100-1,000 ng/ml) for 12 h, after which the culture medium and MTT solution was added and the cells incubated for 4 h. Absorbance was measured at 570 nm using an ELISA microplate reader (Labsystems Multiskan MCC/340, Labsystems, Finland).

Immunohistochemistry
Immunohistochemistry was performed as previously described (42). Animals were anesthetized with 3% isoflurane in nitrogen and oxygen. Then, control, Tat-SOD (50 μg) and BBA (100 μg/ml) combined with Tat-SOD were topically applied onto shaved areas of mouse dorsal skin for 1 h. Frozen sections of skin tissues were prepared and fixed with 4% paraformaldehyde for 10 min, followed by incubation with rabbit anti-histidine IgG (1: 500) for 24 h at room temperature and for 1 h with biotinylated goat anti-rabbit IgG (Vector Laboratories, USA; dilution 1: 200). The sections were visualized with 3,3’-diaminobenzidine (40 mg DAB/0.045% H2O2 in 100 ml PBS) and mounted on gelatin-coated slides. Immunoreactions were observed using an Axioscope microscope (Carl Zeiss, Germany).

Acknowledgements
This work was supported by a Priority Research Centers Program grant (2009-0093812) and by a Regional Research Universities Program/Medical & Bio-material Research Center grant through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

REFERENCES


