細胞外細胞の膠芽腫治療への応用

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The therapeutic applications of extracellular vesicles in glioblastoma

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Glioblastoma (GBM) cells use multiple ways of communication with the tumor microenvironment to tune it for their own benefit. Among these, extracellular vesicles (EV) have emerged as a focus of study in the last few years. Polyphenols and saponins are reported to have many advantages for anti-cancer effects such as high accessibility, low toxicity, and specificity of response, but have limited usage in clinics because of their poor bioavailability and rapid metabolism. Therefore, it would be useful to find a new drug delivery system to improve the bioavailability of natural products in vivo. In this paper, we present on the possibility of using a variety of steroid saponins to treat GBM cells with the purpose of increasing the autophagic potential thereby resulting in an increase of EV production. As an end result, we concluded that the pathway of action differs depending on the concentration. Steroid saponins may have increased EV production by activating autophagy through acid β-glucosidase (GBA) activation.

KEYWORDS: extracellular vesicles, autophagy, steroid saponins.

1. Introduction

Glioblastoma multiforme is the most common glioma and a lethal primary tumor of the central nervous system, characterized by high invasive potential, marked cellular heterogeneity, recurrent disease, and resistance to therapy. In addition, neuroinflammation is also increased after treatment, making the development of alternative therapeutic approaches critically important. [1)]

Autophagy is a highly conserved catabolic process that allows cells to recycle organelles and long-lived intracellular proteins. Induction of autophagy can protect or kill metabolically active cancer cells, including glioblastoma cells, depending on the cellular microenvironment. Even after the "double face" of autophagy was recognized, induction of autophagy has been proposed as another potential anti-tumor mechanism to combat several cancers, including glioblastoma. Recent studies have highlighted common molecular patterns and regulatory pathways between autophagy and extracellular vesicle (EV) biogenesis, suggesting that these processes are closely related.[2)]

For several years, our group has focused on the role of inflammatory processes in the biology of glioblastoma. In particular, steroidal saponins are compounds associated with anti-inflammation and have tumor suppressive functions in several cancers, including glioblastoma. In this regard, a significant upregulation of autophagy is demonstrated in glioblastoma under specific conditions that inhibited the cell proliferation. [2)] In the present study, we provide evidence that the anti-inflammatory agent steroid saponin can induce autophagic flux and EV secretion of glioblastoma.

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2. Materials and Methods

Chemicals and reagents:

Dioscin, rapamaycin, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT dye), and dimethyl sulfoxide (DMSO) were obtained from WAKO. DAL Green was purchased from Dojindo (DALGreen -Autophagy Detection). Coreajaponin was isolated from rhizome of *Dioscorea nipponica* by the method reported by Kim *et al*.[3)]

Cell cultures:

Glioblastoma cell line (A172) was obtained from RIKEN Cell Bank. The cells were maintained in RPMI-1640 medium w/ L-glutamine (WAKO) supplemented with 10% (v/v) of fetal bovine serum (FBS) and cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 97% air.

Cell Treatment：

After reaching confluence, the cells were treated were treated with dioscin (0.1-10 μM) and coreajaponin (0.1-10 μM).

Preparation of crude enzyme solution:

The cells harvested by centrifuge (12,000 rpm for 3 min.) were frozen at -20℃ and thawed rapidly in distilled water, and was designated as the intracellular enzyme. The extracellular solution was dialyzed overnight against distilled water. The dialyzed solution was designated as the extracellular enzyme.

Bata-glucosidase activity:

Enzyme activities were measured by the activity against 0.25 % salicin. A 0.05-mL sample of an appropriately diluted enzyme solution was added to 0.05 mL of substrate in 0.1 M acetate buffer (pH 4.0), and the mixture was incubated at 30 °C. The reaction was stopped by the addition of a copper reagent, and the released reducing sugar was measured by the Somogyi-Nelson method [9)].

MTT reagent-based cellular viability assay:

The MTT was used to quantitatively assess the viability of the iPS and cardiomyocyte cells. The cells were incubated with 10 % of MTT (37 °C; 24 h). After removing the supernatant, the insoluble formazan crystals were dissolved in 100 μL of acid-isopropyl alcohol. The absorbance in each well was measured at a wavelength of 570 nm, and regarded as cellular viability.

Autolysosome formation activity:

The medium was replaced with a culture medium containing 1 μM DAL Green and further incubated at 37 °C for 30 min. Cellular autolysosome formation activity was visualized with a ZEISS Primovert iLED fluorescence microscope with excitation filters having maximum transmission between 425-475 (EX) and 500-560 (EM) nm. Fluorescence images were analyzed by using ImageJ software.

Preparation of dye for EV:

The Oil Red O (ORO) stock solution was prepared by dissolving 5 mg of dye powder in 1 mL of 100% isopropanol and leaving it overnight at room temperature. The following day, it was filtered through a membrane filter with a pore size of 0.2 μm. 350 μl of the ORO stock solution and 150 μL of double distilled water were mixed to prepare a working solution.

Statistical analysis:

All data are presented as mean ± standard deviation (SD). Differences between groups were compared by t-test. $p<0.05$ values were considered to indicate statistically significant differences.

3. Results and Discussion

3. 1 The effects of saponins on cell proliferation

To investigate whether the proliferation of glioblastoma cell is sensitive to steroid saponin treatments, the cells were treated

with increasing concentrations of steroid saponins for 2 weeks. Coreajaponin reduced the cell viability in a dose- dependent manner at week 1 and time-dependent manner at a concentration of 0.1 μM (Fig. 1). Conclusively, these results indicate that 0.1 μM of coreajaponin and dioscin suppresses the proliferation of glioblastoma cell in vitro. (Fig. 1). Diosgenin, an aglycon of diosin and coreajaponin, shows antitumour effects on glioblastoma cells by inducing differentiation.[4] Cell reduction by coreajaponin and dioscin may be attributed to cell differentiation.

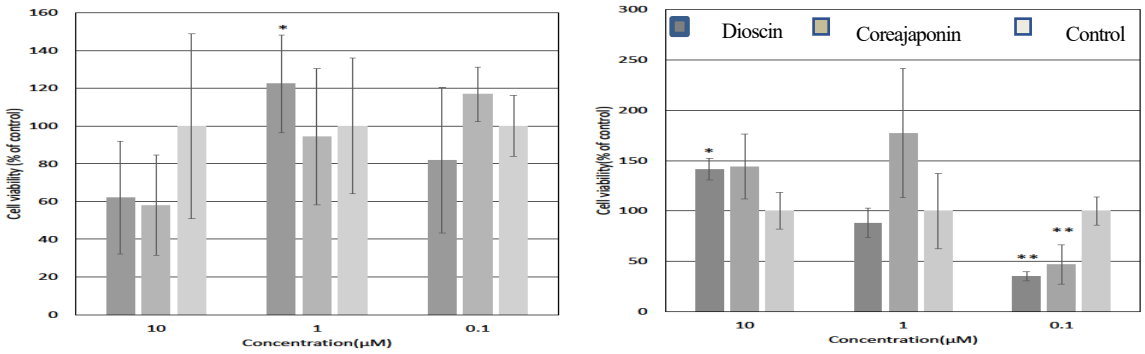

Figure 1 The effects of saponins on cell proliferation. Glioblastoma cells treated by dioscin and coreajaponin were cultivated for 1 week (left) and 2 weeks (right). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *; $p < 0.05$, **; $p<0.01$.

3. 2 The effects of saponins in the autophagic process

The steroid saponin-mediated autophagosome-lysosome fusion was confirmed by fluorescence microscopy (Fig. 2). Both treated and untreated glioblastoma cells were stained with Dal-green reagent. The steroid saponin treatments increased fluorescence intensity, which indicates activation of autophagic fusion in a concentration of 10 μM at week 1, and 0.1 μM at week 2 (Fig. 2). The role of dioscin in autophagy has been well studied, with studies using various tumour cell lines showing that dioscin is active via a PI3k/Akt-dependent or mTOR-dependent pathway and that autophagy induced by dioscin is usually reported to cause cell death. The current study supported that result and found that dioscin was found to cause cell death.[5] On the other hand, coreajaponin induces NIX-mediated autophagy by inhibiting p38MAPK activation in human osteosarcoma cells. [6]

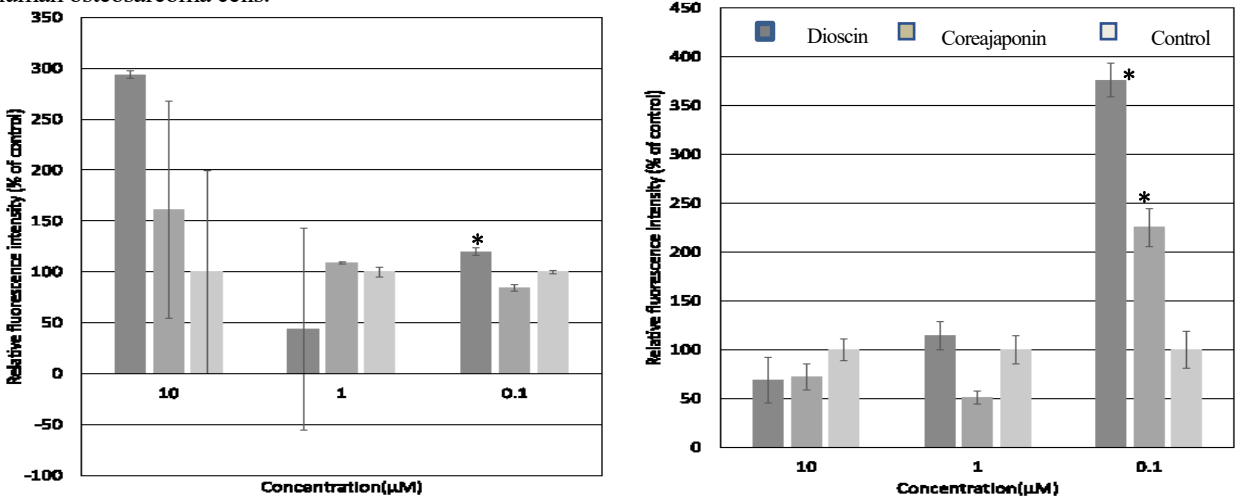

Figure 2 The effects of saponins in the autophagic process. Glioblastoma cells treated by dioscin and coreajaponin were cultivated for 1 week (left) and 2 weeks (right). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *; $p < 0.05$, **; $p<0.01$.

3．3 The effects of saponins on GBA activity in glioblastoma cells

Beta-glucosidase is a lysosomal glycosidic hydrolytic enzyme that catalyzes the hydrolysis of aryl and alkyl-ß-D-glucosides, as well as of glucosides with only the sugar chain portion. Loss of this enzyme leads to lysosomal storage disease, in which lipid derived products and abnormal proteins accumulate. Extensive lysosomal dysfunction not only affects all autophagy pathways and cellular proteostasis, but also has important consequences for other systems in which lysosomal function plays an important role, such as endosomal and exocytotic pathways. [7] Treatment of dioscin increased GBA activity at a concentration of 10μM and 0.1 μM at week 1, and at a concentration of 1 μM and 0.1 μM at week 2 (Fig. 3). Treatment of coreajaponin increased GBA activity at a concentration of 10 μM at week 1, and at a concentration of 0.1 μM at week 2 (Fig. 3).

Pharmacological chaperone (PC) therapy has been proposed and studied as a treatment for many genetic diseases caused by misfolded or unstable proteins, including LSD. Small molecule PCs are designed to selectively bind to and stabilize mutant proteins to promote proper folding and intracellular trafficking, thereby increasing the total amount and activity of intracellular enzymes[8]. Human cytosolic glucosidase shows significant activity and relatively high affinity and specificity for steroid glucosides. Thus, steroid saponins may bind as a substrate and stabilize ß-glucosidase as a molecular chaperone, which may explain the high enzyme activity maintained.

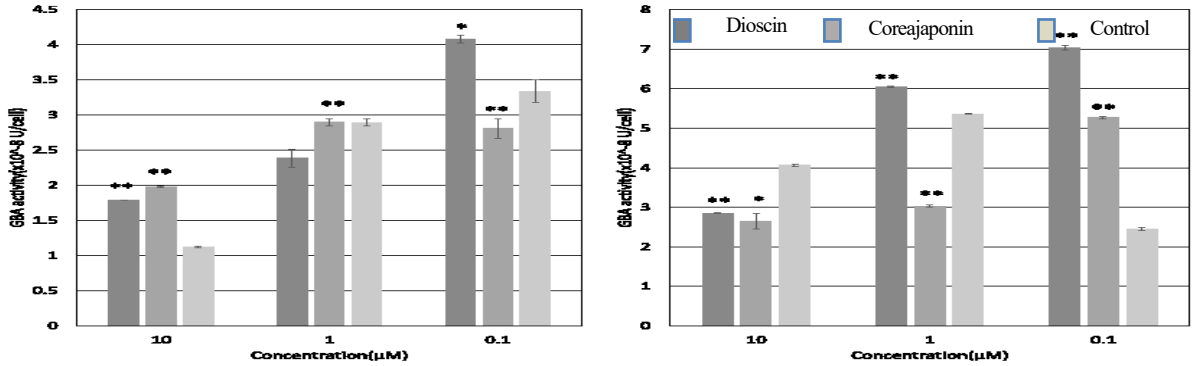

Figure 3 The effects of saponins in GBA activity. Glioblastoma cells treated by dioscin and coreajaponin were cultivated for 1 week (left) and 2 weeks (right). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *; $p < 0.05$, **; $p<0.01$.

3．4 The effects of saponins on EV production of Glioblastoma cells

The total number of EVs resulted in being strongly enhanced after treatment with the steroid saponins at a concentration of 10 μM at week 1, and at a concentration of 0.1 μM at week 2, with a % increase >150% (Fig. 4).

Autophagic processes have many implications in relation to their multiple cellular functions. Although it is possible to prevent the progression of glioblastoma by inducing apoptosis, alternative pathways, such as non-apoptotic autophagic cell death, are equally or more effective in causing the death of glioblastoma cells. Notably, cells with defects in non-functional apoptotic pathways can undergo autophagic cell death. Autophagy has often been reported to be triggered prior to apoptosis, thus making cells more susceptible to death. However, the complex interplay between autophagy and apoptotic mechanisms remains controversial and not fully understood. In particular, the prognosis of glioblastoma may be affected positively or negatively by autophagy. Notably, autophagy may limit the tumour-associated inflammatory profile through the removal of damaged mitochondria as well as inflammasomes, which are thought to be important in supporting the inflammatory

microenvironment that determines tumour progression and invasion. Activation of autophagy is also associated with impaired migration and invasion of glioblastoma cells, which may be reversed and stimulated by inhibition of autophagy.[2)]

Based on these findings and with the aim of highlighting the functional role of steroidal saponins in glioblastoma biology, we explored the possible involvement of autophagic processes and associated vesicle generation in the in vitro antitumour action of steroidal saponins. Under our experimental conditions, steroidal saponins were able to cause a greater induction of autophagic vacuoles and an increase in autolysosome generation. Our findings also suggest that autophagy flux induced by steroidal saponins is associated with GBA activity and cell cycle arrest. We also provide evidence that autophagy induced by steroidal saponins was accompanied by a marked increase in EV secretion by glioblastoma. Furthermore, the activity of GBA, an enzyme involved in EV budding by converting glycosylceramide to ceramide [9)], was significantly increased in glioblastomas after addition of steroidal saponins (Fig. 3).

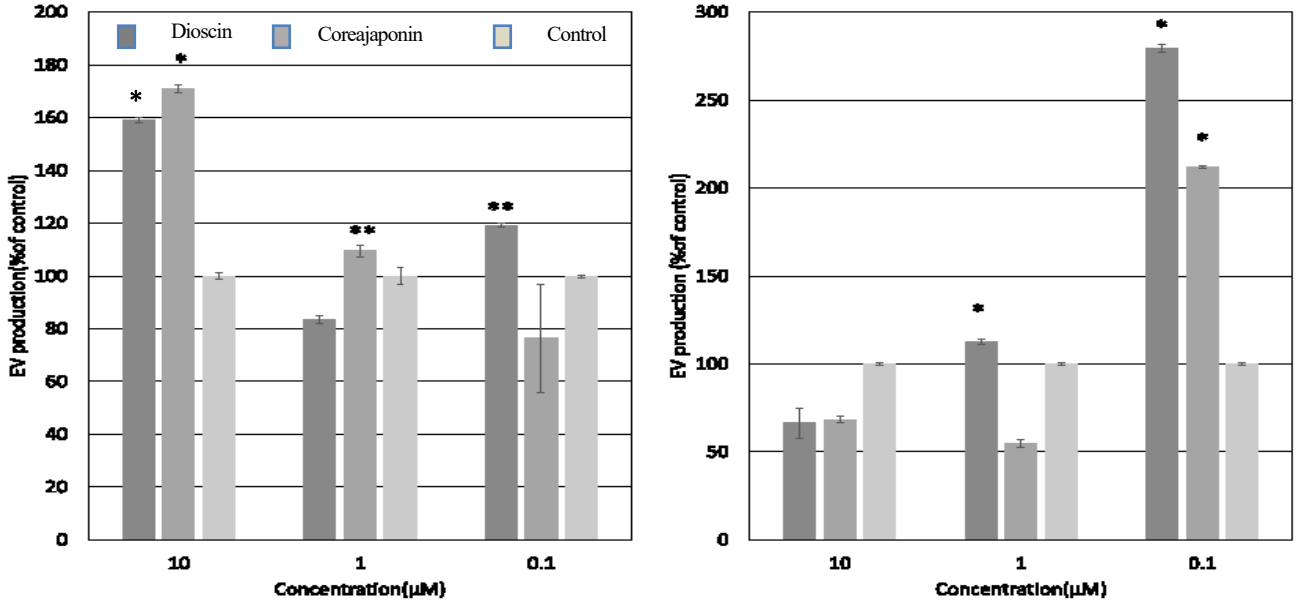

Figure 4 The effects of saponins in EV production. Glioblastoma cells treated by dioscin and coreajaponin were cultivated for 1 week (left) and 2 weeks (right). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *; $p < 0.05$, **; $p<0.01$.

4. Conclusion

Taken together, these findings lead to the hypothesis that the effects induced by the direct addition of steroidal saponins to glioblastoma stem cells may be transmitted to surrounding receptor cells via EVs to deliver the anti-tumour molecular message induced in the origin cell. Autophagy, on the other hand, may induce the release of EVs with modified cargoes that can be manipulated to alter the tumour inflammatory microenvironment to the detriment of tumour growth and invasion.

5. Acknowledgments

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