

Ginseng Saponin as an Antagonist for Gap Junctional Channels

Seung-Keun Rhee[#]

Department of Biochemistry, College of Science, Yeungnam University, Gyongsan 712-749, Korea
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Abstracts : Gap junctional channels, allowing rapid intercellular communication and synchronization of coupled cell activities, play crucial roles in many signaling processes, including a variety of cell activities. Consequently, a modulation of the gap junctional intercellular communication (GJIC) should be a potential pharmacological target. In the present, the GJIC of a epithelial-derived rat mammary cells (BICR-M1Rk) was assessed in the presence of ginseng saponin, by using an established method of scrape-loading dye transfer assay. The transfer of Lucifer yellow (diameter: 1.2 nm) among the neighboring BICR-M1Rk cells, in which connexin43 (Cx43) is a major gap junction channel-forming protein, was significantly retarded at a concentration of 10 µg/ml ginseng saponin. By using both methods of RT-PCR and Western blotting, it was demonstrated that ginseng saponin modulated neither the mRNA synthesis of Cx43 nor the translational process of Cx43. This ginseng saponin-induced modification of GJIC was a similar phenomenon observed under the β-glycyrrhetic acid treatment, a well-known gap junction channel blocker. Taken together, it is reasonable to conclude that the ginseng saponin inhibits GJIC only by modulating the gating property of gap junction channels.

Key words : ginseng saponin, gap junctions, connexin43, RT-PCR, scrape-loading dye transfer assay

1. INTRODUCTION

Gap junctions, as specialized plasma membrane structures, allow the diffusion driven transfer of small hydrophilic cytoplasmic molecules and ions between interconnected cells. The gap junction-mediated intercellular communication (GJIC) influence cellular growth and differentiation, metabolic homeostasis and the synchronization of electrical activity.^{1,2)} Existing models of gap junction channel structure suggest that plasma membranes of the apposing cells that form gap junctions contain hemichannels (also called as 'connexons').³⁾ Hemichannels consisting of hexameric gap junctional proteins (connexins) are pre-assembled in cytoplasmic membranes and transported to the cell membrane by vesicle trafficking.^{4,5)}

The root of *Panax ginseng* has been widely used as restorative drug, sedative, psychic energizer, and an agent to counter senile changes and to prolong vital force in oriental countries.⁶⁾ Recently, detailed studies on ginseng have shown that it has various physiological activities in many cell types, including neuron and smooth muscle cells. Ginseng has shown to contain components similar

to nerve growth factor and epidermal growth factor.^{7,8)} In addition, other experimental evidences indicated that ginseng saponin modulated cell division, tumor metastasis, and smooth muscle contraction.⁹⁻¹¹⁾ Ginseng saponin is also capable to modulate many ionic channels and receptors, including Na⁺ channel, Ca²⁺-activated chloride channel, glycine and acetylcholine receptor.¹²⁻¹⁵⁾

In this work the effect of ginseng saponin on the gap junction-mediated intercellular communication (GJIC) was studied by using a method of scrape-loading fluorescent dye (Lucifer yellow) transfer assay in the cultured BICR-M1Rk cells, which express Cx43 as a major gap junctional channel-forming protein.¹⁶⁾ This study also assessed any changes on the Cx43 expression in the presence of ginseng saponin, using both RT-PCR and Western blotting.

2. MATERIALS AND METHODS

2.1. Cell culture

The rat BICR-M1Rk cell-line (derived from epithelial cells in rat mammary tumor tissue) was routinely maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (BCS) and 10 units/ml penicillin and 10 µg/ml

[#]To whom correspondence should be addressed.
(Tel) +82-53-810-3031; (Fax) +82-53-810-4619
(E-mail) skrhee@yu.ac.kr

streptomycin at 37°C in 5% CO₂ humidified incubator.¹⁶⁾ Ginseng total saponin (GTS) used in this study was kindly provided by Korea Tobacco and Ginseng Corporation.

2.2. Immunofluorescent microscopy

Immunofluorescent labeling was performed as described earlier.¹⁷⁾ Briefly, cells were cultured on polylysine-coated glass cover slips for overnight in 4°C, and then fixed with absolute ethanol. Fixed and cover slip-attached cells were blocked in PBS-2% BSA for 1 hr at room temperature (RT), and then incubated with rabbit anti-Cx43 antibody (Zymed Laboratories) for 1 hr at RT. Cover slips were washed with PBS for three times and incubated with goat anti-rabbit secondary antibody conjugated to CY3 (Amersham Pharmacia Biotech.) for 1 hr at RT. Cover slips were then washed with PBS, dried, and sealed with cover glass. The staining was visualized and photographed with an Axiovert 25-inverted microscope (Zeiss).

2.3. GJIC assay

Cells cultured on fibronectin-coated cover slips were assessed for gap junction-mediated intercellular communication as described previously.¹⁸⁾ Briefly, the culture medium from confluent cells was removed and saved. The cells were rinsed three times with Hanks' balanced salt solution containing 1% bovine serum albumin, after which a 27-gauge needle was used to create multiple scrapes through the cell monolayer in the presence of Dubecco's phosphate-buffered saline containing 0.5% Lucifer yellow. After 3 min incubation at room temperature, the culture was rinsed three times with Hanks' balanced salt solution containing 1% bovine serum albumin and then incubated for additional 8 min in the saved culture medium to allow the loaded dye transfer to adjoining cells. Cells were rinsed and fixed with 4% paraformaldehyde, and viewed using an Axiovert 25-inverted microscope under UV light source.

2.4. RT-PCR analysis

Total RNA was separately obtained from each step of treatments, by suspending the cells in Trizol (Life Technologies) in accordance with the manufacturer's recommendation. Single-strand cDNAs were prepared from 1 µg of total RNA in the presence of avian myeloblastosis virus reverse transcriptase (Promega). The cDNAs of Cx43 were amplified from the cDNA library with a polymerase chain reaction, using following primers: CAGACATGGGTGACTGGAGT as a Cx43 upstream primer, CCGGTTTAAATCTCCAGGTC as a Cx43 downstream

primer. The PCR procedure consisted of 30 cycles of amplification with each cycle consisting of 1 min at 94°C, 1 min at 51°C, and 1 min at 72°C, followed by a final extension for 7 min at 72°C before cooling to 4°C. PCR products were separated by electrophoresis in a 75 constant voltage field in 1.2% agarose gel containing ethidium bromide.

2.5. Western blotting analysis

As described previously,¹⁹⁾ 25 µg total protein extract from plasma membrane fraction of each clone was loaded onto a 10% gel for Cx43 protein. After electrophoresis, proteins were transferred to PVDF membranes (Bio-Rad). The blots were incubated with rabbit polyclonal anti-Cx43 (1/1000; Zymed laboratories), anti-GFP antibodies. Each immuno-reactive band was detected using the ECL system (Amersham). A horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham) was diluted 1:5000 to detect the above primary antibodies.

3. RESULTS

3.1. Identification of Cx43 of gap junctions by immunofluorescent microscopy

Direct immunofluorescence was used to identify the presence of Cx43 at junctional region of MICR-M1Rk cells. The phase-contrast image of cells cultured on polylysine-coated glass cover slips was observed (Fig. 1A). As shown in Fig. 1B, most of the immuno-positive spots for Cx43 were mainly observed either at the cell-to-cell junctional regions or on the plasma membranes, with minor spots on the cytoplasmic area. This observation suggests that the Cx43 expressed in the MICR-M1Rk cells is mainly involved in forming gap junctions. In order to make sure that the immunofluorescent diagram shown in Fig. 1B is not obtained from a non-specific interaction of antibody to protein, rather than Cx43, the cells were directly incubated with CY3-conjugated goat anti-rabbit IgG, omitting an anti-Cx43 primary antibody (Fig. 1C). Consequently, as expected, immunofluorescent spots were not found in the regions of cellular junctions.

3.2. Functional analysis of gap junction channels

A scrape-loading dye transfer method was employed to assess gap junction-mediated intercellular communication (GJIC).¹⁸⁾ Lucifer yellow was chosen as a fluorescent dye for the dye transfer method because it is able to diffuse from one cell to the adjacent cells only through gap junctional channel due to its unique large diameter (1.2 nm).²⁰⁾

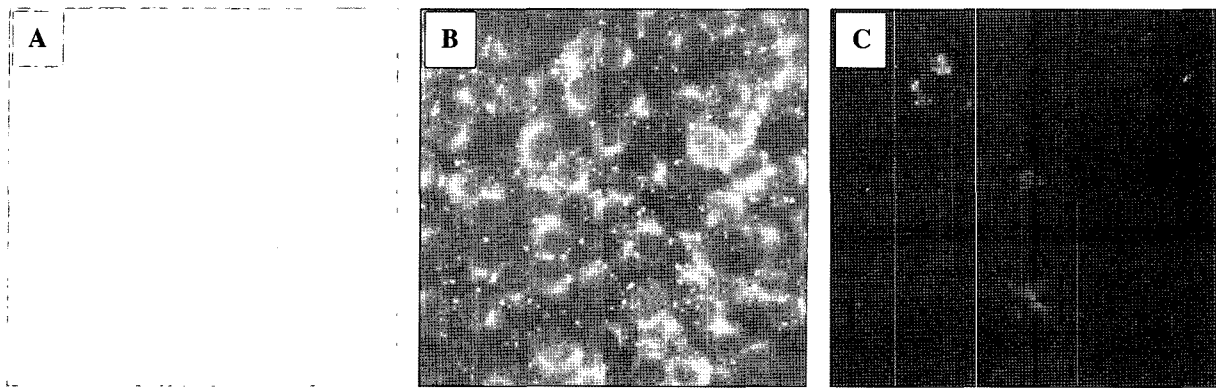


Fig. 1. Immunofluorescent microscopy for Cx43. Panel A: a phase-contrast image of MICR-MIRk cells, Panels B: the immunofluorescent image of the same cells, using both anti-Cx43 primary antibody and a secondary antibody of CY3-conjugated goat anti-rabbit IgG. Panel C: the immunofluorescent image of same cells as shown in panel B, only in the absence of anti-Cx43 antibody, for assessing any non-specific binding of antibody. The fluorescent images were viewed on an Axiovert 25-inverted microscope under UV light source.

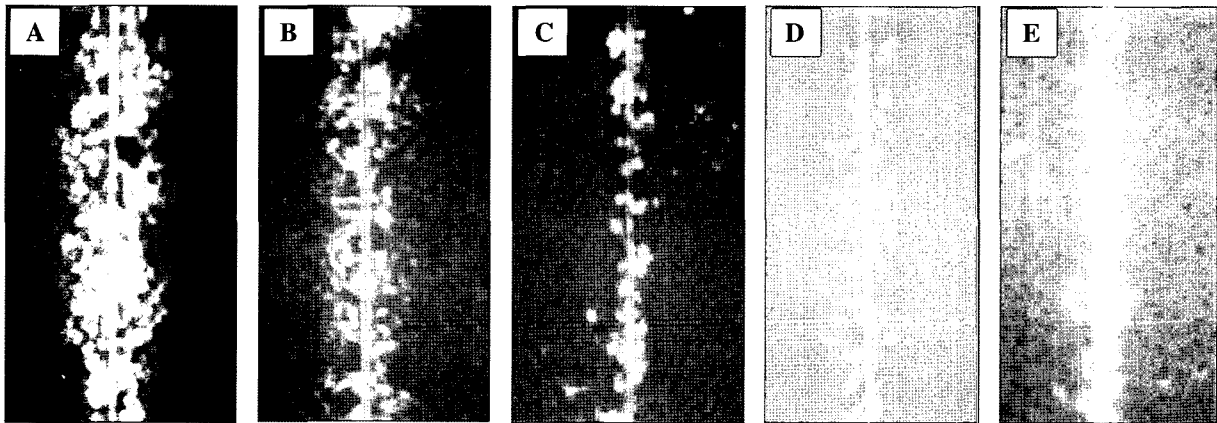


Fig. 2. Effect of ginseng saponin on the gap junction-mediated intercellular communication (GJIC). For the functional analysis of gap junctional channels, both control and ginseng saponin-treated cells for 15 min were subjected to a scrape-loading dye transfer assay, using Lucifer yellow (diameter: 1.2 nm) as dye. Panel A: control cells, Panels B, C, D: cells treated with 1, 10, and 100 $\mu\text{g/ml}$ concentrations of ginseng saponin, respectively. Panel E: cells treated with β -glycyrrhetic acid (0.5 %, w/v), a well-known antagonist for gap junctional channel.

In control cells, the Lucifer yellow was diffused through up to 6 ~ 8 neighboring cells (Fig. 2A). The degree of the Lucifer yellow transfer among cells treated with ginseng saponin appeared to decrease in a dose-dependent manner (Fig. 2B, 2C, 2D corresponding to 1, 10, and 100 $\mu\text{g/ml}$ of ginseng saponin, respectively). As shown in Fig. 2C, 10 $\mu\text{g/ml}$ ginseng saponin significantly retarded the dye transfer among neighboring cells. On the other hand, the Fig. 2E shows a pattern of Lucifer yellow transfer among control cells treated with β -glycyrrhetic acid (5 mg/ml), a well-known potent gap junction channel blocker.²¹⁾

3.3. Effect of ginseng saponin on the Cx43 expression

In order to identify whether the decreased rate of Lucifer yellow transfer through gap junction channels is due to a down-regulation by ginseng saponin for the Cx43 expression, we performed two experiments, reverse transcription (RT)-PCR and Western blotting. The upper and lower panels of Fig. 3 are the results obtained by incubating cells with different concentrations of 1, 10, and 100 $\mu\text{g/ml}$ of ginseng saponin (lanes 3, 4, 5) for 15 min and 1 hr, respectively. As control we treated cells with β -glycyrrhetic acid (5 mg/ml) and compared its effect on mRNA synthesis of Cx43 (lane 6) with that of control

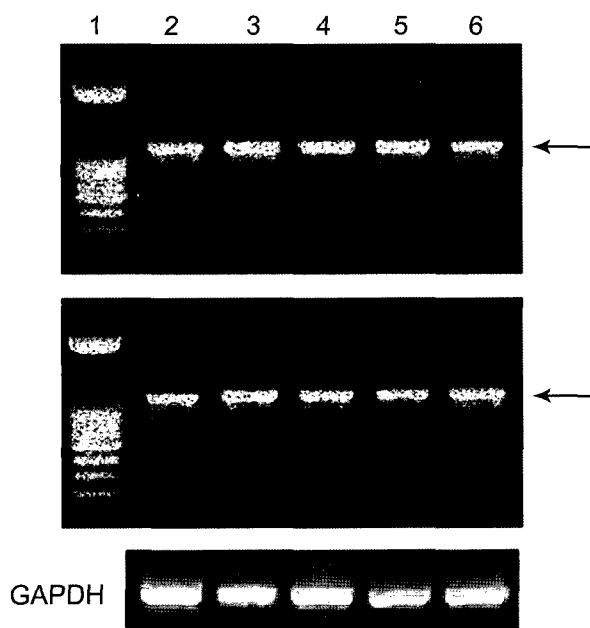


Fig. 3. The effect of ginseng saponin on the mRNA synthesis of Cx43. Total RNAs extracted (25 µg/lane) from cultured cells that were treated with various concentrations of ginseng saponin for 15 min (upper panel) and 1 hr (lower panel) were analyzed by an RT-PCR using rat Cx43 as a primer design. The first two lanes of each panel represent 100 bp DNA ladder (size marker) and cDNA of Cx43 from control cells, respectively. Following lanes (lane 3, 4, 5) represent Cx43 cDNAs extracted from cells that were exposed to 1, 10, and 100 µg/ml of ginseng saponin, respectively. Arrows indicate the position for the Cx43 cDNA. Cells were treated with β-glycyrrhetic acid (5 mg/ml) (lane 6), a potent antagonist for gap junctional channel, and its effect was compared with that of control cells (lane 2).

cells (lane 2). The results obtained by RT-PCT clearly show that there are no remarkable changes on the mRNA syntheses of Cx43 upon ginseng saponin treatment.

Western blotting analysis was performed on the isolated plasma membrane fractions prepared from BICR-M1Rk cells, in order to observe any modification exerted by ginseng saponin on the translation process of Cx43. The cells treated with concentrations of ginseng saponin from 1 to 100 µg/ml were subjected to Western-blotting. As shown in Fig. 4, the treatment of ginseng saponin did not affect the Cx43 expression at protein level. As observed for the mRNA synthesis of Cx43 under a RT-PCR experiment, all the immuno-reactive bands of Cx43 from the ginseng-treated cells were appeared to equal in their strength. The over-all bands strength observed in the cells incubated for

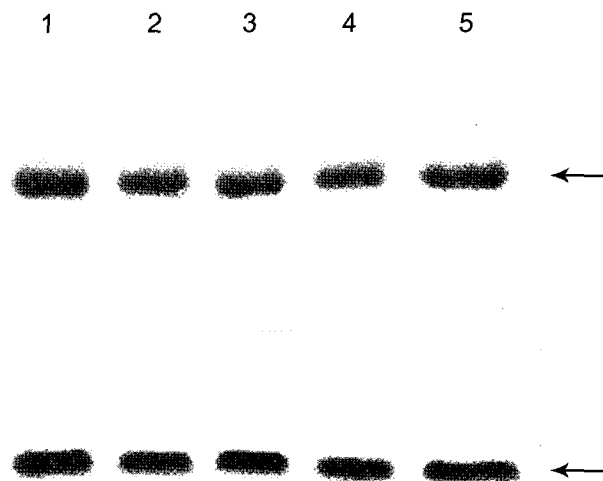


Fig. 4. The effect of ginseng saponin on the Cx43 protein synthesis. Total protein extracted from the plasma membrane fractions of cells that were treated with various concentrations of ginseng saponin was subjected to Western blotting. Upper and lower panels show the amounts of Cx43 present in the plasma membrane fractions of cells that were treated with various concentration of ginseng saponin for 15 min and 1 hr, respectively. Lane 1: control cells, Lanes 2, 3, 4: plasma membrane fractions of cells treated with 1, 10, and 100 µg/ml ginseng saponin. Lane 5: plasma membrane fraction of cells treated with β-glycyrrhetic acid (5 mg/ml), a potent antagonist for gap junctional channel. Arrows indicate the positions of the immuno-reactive bands for Cx43.

1 hr with ginseng saponin (lower panel of Fig. 4) was a little weaker than those observed in the cells incubated for 15 min (upper panel). In summary, the experimental results obtained from RT-PCR and Western-blotting clearly demonstrated that the ginseng saponin does not affect on the synthesis of Cx43 in BICR-M1Rk cells, neither in transcriptional nor in translational process.

4. DISCUSSION

Gap junctional channels have been shown to be involved in important cellular activities, as well as in pathogenesis of many inherited and acquired human diseases.¹⁻²⁾ Therefore, agents that regulate gap junction-mediated intercellular communication (GJIC) should be able to agonize or antagonize various cell functions. The present study examined

the effects of ginseng saponin isolated from *Panax ginseng* on GJIC. The inhibitory effect of ginseng saponin on the GJIC was significant and dose-dependent. The GJIC between BICR-MIRk cells was completely abolished with a concentration (or higher) of 10 $\mu\text{g/ml}$ ginseng saponin, as compared with a well-known potent GJIC blocker, 18 β -glycyrrhetic acid.²¹⁾ In previous study a similar inhibitory effects of ginseng saponin was observed in a reconstituted gap junction hemi-channel system.²²⁾ The sucrose-permeability of the reconstituted gap junctional hemi-channel was decreased upon treatment of ginseng saponin by 50% at a concentration of 10 $\mu\text{g/ml}$. However, the author was not able to identify whether the ginseng-induced inhibition is reversible or not since it was impossible to wash out all the traces of ginseng saponin sufficiently enough.

The results obtained from the both experiments of RT-PCR and Western blotting ruled out the possibility of down-regulation induced by ginseng saponin on the Cx43 synthesis, which could lead to a reduced GJIC. In general, the concentrations at which ginseng saponin components affect various channels and receptors are in ten micromolar range. The EC_{50} value for the increase of Ca^{2+} activated Cl^{-} currents in *Xenopus* oocytes by ginseng saponin was around 30 μM .²³⁾ The concentration of a ginsenoside, Rg2, that reduced acetylcholine-evoked Ca^{2+} and Na^{+} influx in bovine chromaffin cells was also in the same concentration range.²⁴⁾ Other ginsenosides, such as Rf for example, also affected Ca^{2+} channels with an IC_{50} of 40 μM .²⁵⁾ Similarly, this present study showed that GJIC was almost completely inhibited by ginseng saponin at the concentration of 10 $\mu\text{M/ml}$, which is approximately equivalent to 10~20 μM . if considering the average molecular weight of ginseng saponin as 620 Da.²⁶⁾

In conclusion, the results obtained from this work showed that ginseng saponin has an antagonistic activity for the gap junction channels, principally altering gating of the channels. Because gap junction-mediated cellular communication is important for the process of carcinogenesis, there may be many consequences of manipulation of gap junction channel gating. According to an array of previous studies, ginseng saponin or various ginsenosides have been reported to have suppressive effect on the growth of various cancer cells.²⁷⁻²⁹⁾ Therefore, the inhibitory effect of ginseng saponin on GJIC observed in this study could generate considerable interest, and the modulation of GJIC by ginseng saponin could be one of the mechanisms of its variable pharmacological and therapeutic properties.

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