Stimulatory Effects of Ginsan on the Proliferation and Viability of Mouse Spleen Cells

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Ginsan is an acidic polysaccharide purified from *Panax ginseng*, a famous oriental herb. Although a variety of biological activities of ginsan have been studied, the effects of ginsan on spleen cells are not fully elucidated. We investigated the effect of ginsan on the viability and proliferation of spleen cells. Using Cell Counting Kit-8® solution and trypan blue solution, we found that ginsan significantly enhanced viability and proliferation. Multiple clusters, indicating proliferation, were observed in ginsan-treated spleen cells and, carboxyfluorescein succinimidyl ester and surface marker staining assay revealed that ginsan promoted proliferation from CD19+ B cells rather than CD4+ or CD8+ T cells. In addition, ginsan decreased the percentage of late apoptotic cells. Ginsan increased the surface expression of CD25 and CD69 as well as production of interleukin-2 from spleen cells, suggesting increased activation. Taken together, these results demonstrate that ginsan increases the viability and proliferation of spleen cells via multiple mechanisms, valuable information for broadening the use of ginsan in clinical and research settings.

Key Words: B lymphocytes, Ginsan, Stimulation, Proliferation, Spleen cells
cells/200 μl/well in 96-well culture plates for a cell viability/proliferation assay, using Cell Counting Kit-8® solution (Dojindo, Gaithersburg, MD). Briefly, the cultured wells were treated with 10 μl/well of Cell Counting Kit-8® solution for last 4 hr and the optical density of wells was measured at 450 nm using a microplate reader (Molecular devices, Sunnyvale, CA). For measuring cell viability, the treated spleen cells in 96-well culture plates were stained with a trypan blue solution (Invitrogen, Carlsbad, CA) and the viable and dead cells counted. The pictures of cultured cells were taken by digital camera (Cannon Inc., Tokyo, Japan) connected to inverted microscope (Olympus Co., Tokyo, Japan).

Flow cytometric analysis

The spleen cells were cultured at a concentration of 5×10^6 cells/5 ml/well in 6-well culture plates in the absence or presence of ginsan for 3 days for flow cytometric analysis. The staining of cells was processed as established in our laboratory [2,9]. Spleen cells were treated with biotin-labeled anti-CD4, anti-CD8, anti-CD19, anti-CD25 antibody and then streptavidin-phycoerythrin (PE), or PE-labeled anti-CD69 antibody (all from BD Biosciences, San Jose, CA). For measuring the cell death, both apoptosis and necrosis, spleen cells were stained with annexin V-fluorescein isothiocyanate (FITC)/ propidium iodide (PI) kit (Biosource International) according to the manufacturer's instruction. To check the membrane potential of mitochondria [10], spleen cells were incubated with 10 μg/ml rhodamine 123 (Sigma) for 30 min at room temperature. All stained cells were analyzed by FACS Calibur® and CellQuest® (Beckton Dickinson, Franklin Lakes, NJ). For measuring the proliferation of specific cell subsets, the spleen cells were stained with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min and then cultured in 96-well culture plates with or without ginsan.

Enzyme-linked immunosorbent assay (ELISA)

Spleen cells were cultured as described in the viability/proliferation assay. After 3 day culture, the supernatants were harvested and used for ELISA. The amounts of IL-2 and IL-4 in supernatants were determined by using Cytoset® kit (Biosource International) according to the manufacturer's instruction.

Statistical analysis

Data were presented as mean±SD and statistically analyzed by Tukey-Kramer multiple comparisons test. A p value of <0.05 was considered as significant.

RESULTS

The effect of ginsan on the proliferation and viability of spleen cells

To study the effect of ginsan on the proliferation and viability of spleen cells, we utilized CCK-8 assay and a trypan blue exclusion test. The optical density of spleen cells treated with 0~200 μg/ml ginsan was measured for 4 days. Interestingly, ginsan promoted splenic cell proliferation at 8~200 μg/ml from day 2 through day 4 (Fig. 1A). To de-

![Fig. 1. Effect of ginsan on the proliferation/viability of spleen cells. The spleen cells were cultured at a concentration of 2×10^5 cells/200 μl/well in 96-well culture plates. (A) Proliferation assay using Cell Counting Kit-8® solution, in which spleen cells were treated with 0~200 μg/ml ginsan for 4 days. (B) Trypan blue exclusion test, in which spleen cells were treated with 0~100 μg/ml ginsan for 3 days. Data are mean±SD from three or four individual wells.

![Fig. 2. Proliferating clusters in ginsan-treated spleen cells. Spleen cells were cultured as described in Fig. 1, and treated with medium alone, 10 μg/ml or 50 μg/ml ginsan for 3 days. (A) Cell morphology was observed using an inverted microscope and the image was obtained by a digital camera. (B) Cell size of the treated spleen cells was analyzed by flow cytometry.
termine the effect of ginsan on the viability of spleen cells, we used trypan blue staining. The viability of spleen cells treated with ginsan was significantly greater than controls at concentrations of 10 and 100 μg/ml (Fig. 1B). Clusters of proliferating spleen cells treated with ginsan were visualized by an inverted microscope (Fig. 2A). Based on cell size analysis (Fig. 2B), ginsan profoundly increased the percentage of blasting cells located in a given region (R1). Thus, the results of proliferation and viability assays suggest that ginsan has some stimulatory effects on spleen cells. To further investigate which subset of spleen cells proliferate, we utilized CFSE to identify dividing cells after ginsan treatment for 3 days (where the CFSE intensity of proliferating cells gradually decreases upon repetitive divisions). CD19⁺ B lymphocytes selectively proliferated with ginsan treatment, whereas CD4⁺ and CD8⁺ T lymphocytes did not (Fig. 3).

**The protective effects of ginsan on spontaneous cell death of spleen cells**

Harvested spleen cells gradually enter a spontaneous cell death process due to the absence of the growth cytokines that in vivo provide survival and proliferating signals [11-13]. To investigate whether ginsan may protect spleen cells from spontaneous cell death in vitro, we stained cells with annexin V-FITC and PI to measure cellular apoptosis and necrosis. Indeed, as shown in Fig. 4, ginsan protected spleen cells from cell death, as illustrated by the increase of cell percentage in the lower left quadrant and the decrease of cell percentage in the upper right quadrant of the dot figure. To measure the action potentials of the mitochondrial membrane, the cells were stained with rhodamine 123 solution, because apoptotic and necrotic cells have lower action potentials than viable cells. The analysis indicated that ginsan increases the action potentials of the mitochondrial membrane of spleen cells (Fig. 4).

**Enhanced expression of activation markers on ginsan-treated spleen cells**

To determine the effect of ginsan on lymphocyte activation, we measured the surface expression levels of the activation markers CD25 and CD69 on spleen cells following treatment with ginsan. CD25 is an alpha chain of the IL-2 receptor that enhances the sensitivity of lymphocytes to IL-2, and CD69 is an early-activation marker for lymphocytes [14]. Treatment with 50 μg/ml ginsan increased the expression of both activation markers on spleen cells (Fig. 5).
Flow cytometry analysis demonstrated that ginsan increases some activation markers on spleen cells, which may sensitize cells to growth cytokines such as IL-2.

**Ginsan-activated spleen cells respond to IL-2**

To generate an immune response, it is critical to induce the proliferation of lymphocytes, including clonal expansion.

![Image](Fig. 6).

**DISCUSSION**

Ginsan promotes cellular cytotoxicity against a variety of tumor cells [17]. In addition, ginsan-activated killer cells have phenotypes that are distinct from IL-2-activated killer cells. Although previous studies of the effects of ginsan on spleen cells have focused on the function of effector cells, we aimed to elucidate its effects on additional aspects of lymphocyte function, such as which subsets respond to ginsan, the potential protective roles against spontaneous cell death, and influences on activation and related cytokine production.

The spleen consists of T and B lymphocytes, natural killer cells, macrophages, and dendritic cells. The spleen cells used in the present study were almost all lymphocytes, due to the removal of adherent cells during the preparation procedure. This was supported by flow cytometric analysis, which showed that more than 90% of the cells were CD4⁺, CD8⁺, and CD19⁺ cells. Although previous studies have demonstrated that cytotoxic T lymphocytes and LAK cells are generated with ginsan when synergized with IL-2, we observed increased proliferation of CD19⁺ B lymphocytes in ginsan-activated spleen cells suggests that ginsan could be used to enhance humoral immunity [18]. However, it remains unclear which isotype of IgG may be produced by ginsan treatment and whether or not ginsan may regulate the production of antigen-specific IgG. These questions should be investigated in future studies.

Harvested spleen cells enter a state of spontaneous cell death due to the lack of growth and survival factors that are provided in vivo. In the experimental design, ginsan enhanced the viability of spleen cells in a concentration-dependent manner. Therefore, we investigated the mechanism by which ginsan enhances viability, and found that ginsan decreased the percentage of cells in late apoptosis. In addition, we found that spontaneous cell death of spleen cells and the protective effects of ginsan were closely related to the mitochondrial membrane potential.

A variety of immune responses are closely associated with the function of lymphocytes as effector cells. To mount a sufficient immune response, the lymphocytes, especially antigen-specific clones, must proliferate. We demonstrated that lymphocytes proliferated more following ginsan activation plus IL-2 treatment than after IL-2 treatment alone. Because ginsan also increased the expression of CD25, the alpha chain of the IL-2 receptor, ginsan may sensitize...
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spleen cells to IL-2 via CD25 expression. In addition, ginsan treatment enhanced IL-2 production from spleen cell culture in vitro. Taken together, these data strongly suggest that ginsan induces the proliferation of lymphocytes, including clonal expansion, via multiple mechanisms. Ginsan has cytotoxic and proliferative effects on spleen cells, and selectively influences B lymphocyte proliferation. This study may help researchers to broaden the use of ginsan as an immunostimulating agent.

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REFERENCES


