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Enhancement of anti-tumor activity of gamma-irradiated silk fibroin via immunomodulatory effects

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ABSTRACT

Silk fibers have proven to be effective in many clinical applications as well as for clothing. In addition to the substantial effect of silk fibers, the present study was conducted to explore its importance in a new dimension to reinforce the effects of its physiological function regarding anti-tumor activity and immune response with gamma-irradiated silk fibroin (GISF). The cytotoxicity results showed that pre-treatment of GISF in the mouse peritoneal macrophages (MPM) indicated a higher proliferative effect than that of non-irradiated silk fibroin (NISF) in a concentration-dependent manner. Based on the cytotoxicity result of MPM, GISF (50 and 150 kGy) was selected for an *ex vivo* study in an animal (C57BL6) system and evaluated about whether the non-specific immune response was also related to GISF. GISF (50 and 150 kGy) augmented immune responsiveness via activation of NK cells, T lymphocytes proliferation, NO production, and cytokine level, such as IL-6, IL-2, IL-12, IFN-γ, TNF-α, as compared with NISF, which strongly suggested that GISF significantly augmented an important element of all aspects of the innate and adaptive immune system. Therefore, from these results, it seems likely that the GISF will play a potent role in eliciting the effect of the non-specific immune response and anti-tumor activity as a value-added product in the medical industry.

1. Introduction

Silk derived from the silkworm *Bombyx mori* is a natural protein that is made mainly of sericin and fibroin proteins [1]. In the olden days, fibroin was used only for clothing, but silk fibers have proven to be effective in many clinical applications during its decades of use. Fibroin is known as a potential candidate material for biomedical applications such as enzyme-immobilizing membranes [2] and an oral dosage gel form [3]. Several biological activities of fibroin have also been reported; lowering of blood cholesterol and glucose levels and alcohol absorption were observed in fibroin-fed rats [4] and sulphated fibroins were shown as anti-HIV activity agent [5]. Although many efforts have been made to develop and improve immunotherapy strategies for the treatment of malignancies, one of the major problems facing cancer chemotherapy is achieving the specificity of drug action. The key is the administration of the required therapeutic concentration of the drug at the desired tumor site for the desired period of time without causing undesirable side effects on other organs after systemic administration [6].

Recently, it has become clear that gamma irradiation is a useful technology in the safe storage of food and maintenance of food hygiene. Also it has been found to have importance in the medical and beauty care industry [7], but the mechanisms by which gamma irradiation produce several physiological effects, such as anti-tumor activity, are still unknown as well as there being many controversial reports despite the large amount of work regarding the change of structural and physiological properties of protein by irradiation. Our previous report was able to demonstrate that a gamma-irradiated silk fibroin (GISF) reduced the tumor growth in B16BL6 (mouse melanoma cancer) inoculated tumor bearing mouse [8], which strongly suggests that GISF is a useful model to examine the physiological activities. Based on the previous report, the GISF led us to do further experiments of the immune response regarding the preventive effects against tumors and the major objective of our present study was to assess how GISF induces the tumor growth inhibitory effect on an innate and adaptive immune system. Accordingly, this investigation was undertaken to explore the importance of GISF in a new dimension to reinforce its effects of physiological function.

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2. Materials and methods

2.1. Preparation of fibroin

Cocoons were supplied by Nuero Ltd. (Daegu, Republic of Korea). To remove the sericin, 50 g of cocoons was cut into small pieces and boiled in 2.5 L of 5% (w/v) Na_2CO_3 for 1 h and filtered through a filter paper. The remaining sericin and Na_2CO_3 were removed by washing the residue with hot water three times. The fibroin was solubilized using a solution of $CaCl_2$–H_2O–ethanol [9]. A mixture containing 226.4 g $CaCl_2$, 346 g distilled water and 280 mL ethanol was added to 35 g fibroin residue and heated at 100 °C for 2 h. The dialysis (molecular weight over 1000 Da, Spectrum Laboratories Inc., CA, USA) was carried out five times, and then the dialyzed solution was dried in a vacuum freeze dryer to obtain fibroin powder. This powder was stored in a refrigerator at 4 °C for further experimentation.

2.2. Gamma irradiation

Fibroin was dissolved in a concentration of 5 mg/mL (w/v) in deionized distilled water (DDW). Fibroin solution was irradiated at 0, 5, 10, 50, 100, 150, and 200 kGy in a cobalt-60 irradiator (point source AECL, IR-221, MDS Nordion International Co., Ltd., Ottawa, ON, Canada) with an 11.1 PBq (peta-becquerel) source strength and operated at a radiation dose rate of 10 kGy/h. The GISF solution was stored at 4 °C until it was used. Dosimetry was carried out using a 5-mm-diameter alanine dosimeter (Bruker Instruments, Rheinstetten, Germany). The dosimeter was calibrated using International Atomic Energy Agency (Vienna, Austria) standard.

2.3. Experimental animals

Six-week-old female C57BL6 mice (body weight; 17–19 g) were purchased from Orient Inc. (Charles River Technology, Seoul, Korea). For vivarium adaption, the mice were housed in a polycarbonate cage and fed a standard animal diet and water *ad libitum* under controlled temperature conditions (22 ± 2 °C) with 12-h light and dark cycles for a week before the fibroin treatment.

2.4. Macrophage cells culture from mice

Mouse peritoneal macrophages were harvested from thioglycollate medium (Sigma Chemical Co., St. Louis, MO, USA) treated mice as described previously [10]. Macrophages were loaded into petri dishes (100 φ). After 2-h incubation, non-adherent cells were removed by washing with PBS and adherent macrophages were co-incubated with NISF and GISF. Counted cells (1×10^6 cell/well) were re-suspended in complete RPMI-1640 medium and then plated into 48-well culture plates. After 24 h, cultured supernatants were harvested and stored at −70 °C for cytokine productions and cultured pellets were used for macrophage proliferation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co.) assay as described by Zhanga et al. [11].

2.5. Assessment of NO production

NO levels were determined by Griess reagent (Sigma Chemical Co.). Macrophage culture supernatant and Griess reagent were in equal volumes (100 μL) mixed according to manufacturer's instructions. The absorbance was measured at 595 nm using an ELx808 absorbance microplate reader (Zenyth 3100, Anthos Labtec Instruments GmbH, Salzburg, Austria). The absorbance values were then converted to concentrations (μM) of NO using standard curves prepared with serial dilutions of $NaNO_2$ standards.

2.6. Cytokines assay

Cultured supernatants were used for the cytokine production test. ELISA kits (BD Biosciences, San Jose, CA, USA) were used with the splenocyte suspensions according to the manufacturer's instructions, and absorbance for interleukin (IL) -2, IL-6, IL-12, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in splenocyte cultured supernatants were measured at 450 nm with a microplate reader (Zenyth 3100). The absorbance values were then converted to concentrations (pg/mL) of IL-2, -6, -12, IFN-γ and TNF-α using standard curves prepared with serial dilutions of recombinant IL-2, IL-6, IL-12, IFN-γ and TNF-α standards.

2.7. Tumor induction

Experimental tumor induction of tumor cell, B16BL6 (mouse melanoma, 4×10^4 cells) was assessed by intra-dermal (id) injection into C57BL6 mice (4 groups, 5 mice per a group) and tumor non-injected mice were used as a normal control. Tumor control group mice were given oral administration (2.5 mg/head) of PBS, NISF (0 kGy), and GISF (50, 150 kGy) for 7 days. The mice were sacrificed 14 days after tumor inoculation and spleen tissues were separated from the tumor-inoculated mice for measurement of NK cell activation, splenocyte proliferation, cytokine productions, and cell surface cluster of differentiation ($CD4^+$, $CD8^+$) population percentage.

2.8. Splenocytes isolation and culture from tumor bearing mice

Spleen cells from tumor injected mice and normal mice were obtained by gentle disruption of the spleen (C57BL6 & BALB/c mice) and repeated pipetting was done to attain single-cell suspension. Then, the cell suspensions were washed two times with ice cold RPMI-1640 medium developed by Cederbrant et al. [12]. Splenocytes were separated from erythrocytes using ice cold distilled water for 30 s and then counted for cell viability (90%). Splenocytes were plated in 96-well tissue culture plates at a final concentration of 1×10^6 cells/well maintained in complete medium and then cultured at 37 °C in 5% CO_2 incubator [12]. Cultured supernatant harvested after 24 h and stored at −70 °C for cytokine productions and cultured pellets were used for spleen cell proliferation by MTT assay.

2.9. Flow cytometric analysis of NK, $CD4^+$ and $CD8^+$ cell population

The splenocytes from tumor injected mice fed with PBS, NISF (0 kGy), and GISF (150 kGy) for 7 days were re-suspended with PBS containing 0.5% FBS as a final number of 1×10^6 cells/mL. Fifty-fold diluted anti-mouse CD1/32-block FC (eBioscience, San Diego, CA, USA) was added and incubated in 4 °C for 30 min in dark condition. Fluorescein isothiocyanate (FITC)-conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CD8 and PE-Cy7-conjugated anti-mouse pan NK monoclonal antibodies, T cell surface markers, were added to the cells as 1:50 and incubated at 4 °C for 30 min in dark condition. After washing with PBS, cells were fixed in 1% paraformaldehyde. A total of 10,000 cells per run were analyzed on a flow cytometry (Beckman Coulter Co., Fullerton, CA, USA).

2.10. NK cell -mediated tumor cell cytotoxicity

The NK cell-mediated tumor cell cytotoxicity was evaluated by non-radioactivity cytotoxicity kit (Promega Co., San Luis Obispo,