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Short Communication

Recombinant spider silk fibroin protein produces a non-cytotoxic and non-inflammatory response

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ABSTRACT

Silk fibroin proteins serve as biomaterials for diverse applications. Previous studies have shown that silk fibroin proteins can be used in biomedical applications for treating diabetes. In the present study, we investigated the cytotoxicity and inflammatory response induced by spider silk fibroin protein in vitro. By using recombinant AvMaSp-R spider silk fibroin protein, which consists of the 240 amino acid repetitive domain of the spider (*Araneus ventricosus*) silk fibroin protein and was expressed in baculovirus-infected insect cells, we tested the cytotoxicity, apoptosis, macrophage stimulation, and release of proinflammatory mediators and cytokines in vitro. We found that recombinant AvMaSp-R was not cytotoxic and did not activate macrophages. Consequently, our results provide evidence that recombinant AvMaSp-R produces a non-cytotoxic and non-inflammatory response, suggesting that recombinant AvMaSp-R silk fibroin protein could be a biomaterial for biomedical applications.

Introduction

Silk fibroin proteins are attractive biomaterials for biomedical applications because of their unique properties such as biocompatibility, biodegradability, and high tensile strength (Altman et al., 2003; Lewis, 2006; Vepari and Kaplan, 2007; Kluge et al., 2008). Silk fibroin proteins from silkworms or spiders are primarily composed of glycine (G) and alanine (A) residues (Zhou et al., 2000; Ayoub et al., 2007; Lee et al., 2007, 2012). Diverse biological effects of silk fibroin proteins in the biomedical field have been reported, such as preventing DNA damage (Park et al., 2002), reducing blood pressure (Igarashi et al., 2006), anti-human immunodeficiency virus activity (Gotoh et al., 2000), and enhancing the metabolism of glucose (Gao et al., 2000; Hyun et al., 2004; Park et al., 2002; Jung et al., 2010; Do et al., 2012; Lee et al., 2014) and lipids (Jung et al., 2010).

The heavy chain of silkworm silk fibroin contains 5263 amino acid residues and forms 2377 repeats of a GX dipeptide motif in which the X residues are primarily composed of Ala (64%), Ser (22%), and Tyr (10%) (Zhou et al., 2000). Spider silk fibroins contain repetitive motifs such as GPGXX motifs, alanine-rich stretches [$(A)_n$ or $(GA)_n$], and GGX motifs (Scheibel, 2004; Lee et al., 2007, 2012). Recombinant partial fibroin silk fibroin proteins, bioengineered silk proteins, and synthetic silk fibroin peptides have been produced for various biomedical applications (Huang et al., 2010; Lee et al., 2012; Dams-Kozlowska et al., 2013). However, before these silk fibroin proteins with various structures can be used for biomedical applications, it is necessary to elucidate their toxicity and immunogenicity.

Our previous study reported the cloning and expression of partial cDNAs encoding the C-terminus of the spider (*Araneus ventricosus*) major ampullate silk protein (AvMaSp-R), which encodes 240 amino acids with 7 $(A)_n$, 15 $(GA)_n$, and 38 GGX amino acid motifs (Lee et al., 2012). We also found that recombinant AvMaSp-R spider silk fibroin protein enhances insulin secretion and reduces blood glucose levels in diabetic mice (Lee et al., 2014). The objective of the present study was to evaluate the cytotoxicity and macrophage activation of recombinant AvMaSp-R spider silk fibroin protein. We assayed the effects of recombinant AvMaSp-R on cytotoxicity, apoptosis, macrophage stimulation, and the release of proinflammatory mediators and cytokines. We provide evidence that recombinant AvMaSp-R spider silk fibroin protein induces a non-cytotoxic and non-inflammatory response.

Materials and methods

Recombinant AvMaSp-R spider silk fibroin protein

The spider silk fibroin protein used in this study was recombinant AvMaSp-R, as previously described (Lee et al., 2012, 2014). The expression and purification of recombinant AvMaSp-R were performed as previously described (Lee et al., 2012, 2014).

Cytotoxicity and cell viability assays

Cytotoxicity and cell viability assays were performed using NIH 3T3 cells, a murine fibroblast cell line, cultured in Dulbecco's modified

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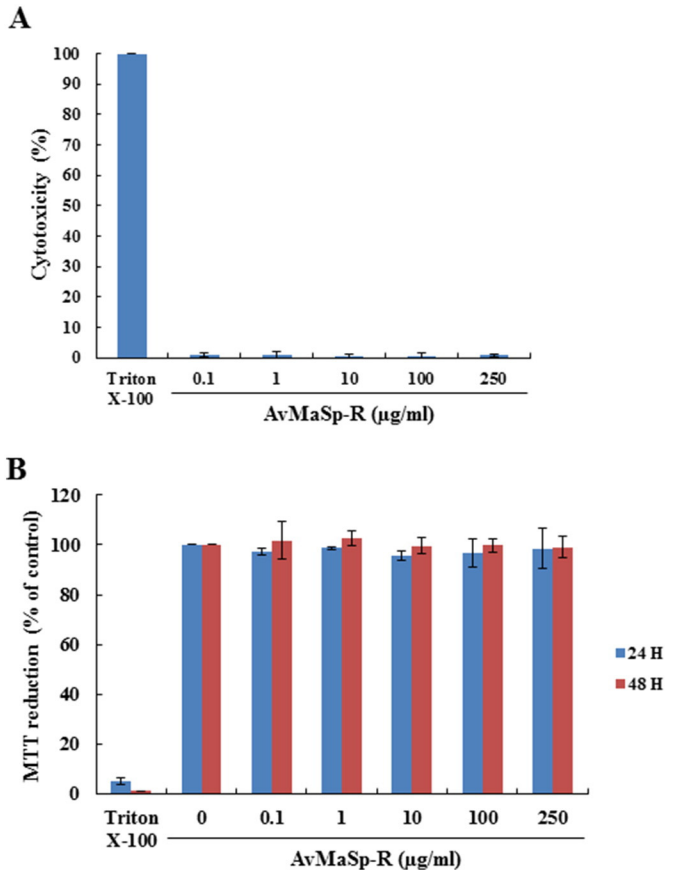

Fig. 1. Cytotoxicity and cell viability of recombinant AvMaSp-R. (A) Cytotoxicity of recombinant AvMaSp-R. Cytotoxicity assays were performed using the murine fibroblast cell line NIH 3T3 with recombinant AvMaSp-R (0.1, 1, 10, 100, or 250 μg per ml of medium) or Triton X-100 (control). (B) Cell viability after treatment with recombinant AvMaSp-R. For the MTT assay, NIH 3T3 cells were treated with recombinant AvMaSp-R (0.1, 1, 10, 100, or 250 μg per ml of medium) or Triton X-100 (control). The percentage of MTT reduction was determined at 24 and 48 h. Bars represent the mean ± SD (n = 3).

Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, USA) at 37 °C in an atmosphere of 5% CO_2. Cells were cultured in 96-well plates (5×10^4 cells/well) for 24 h, and then recombinant AvMaSp-R (0.1, 1, 10, 100, or 250 μg per ml of medium) or 1% Triton X-100 (Sigma) was added to each well. After incubation for 24 h, the harvested media were used for the lactate dehydrogenase (LDH) assay using the LDH-Cytotoxicity Assay Kit II (BioVision, Milpitas, CA, USA) according to the manufacturer's protocol. For the 3-(4,5 dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, NIH 3T3 cells were cultured in 96-well plates (5×10^4 cells/well) with recombinant AvMaSp-R (0.1, 1, 10, 100, or 250 μg per ml of medium) or 1% Triton X-100 (Sigma) for 24 or 48 h, followed by the addition of 50 μl of MTT reagent (BioVision) to each well. After incubation for 4 h, the media were discarded, and the formazan crystals were dissolved in 150 μl of MTT solvent. Absorbance was measured at 590 nm using a microplate reader (Bio-Rad Model 3550, Bio-Rad, Hercules, CA, USA). For cytotoxicity and cell viability assays, the positive control used was 1% Triton X-100. Data are presented as the mean ± standard deviation (SD) of three replicates.

Apoptosis assay

Apoptosis was evaluated by measuring the caspase 3 activity using the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's protocol. NIH 3T3 cells were cultured in 6-well plates (1×10^6 cells/well) for 24 h, and then recombinant AvMaSp-R (0.1, 1, 10, 100, or 250 μg per ml of medium) was added to each well. After incubation for 24 h, cells were harvested, washed with phosphate-buffered saline (PBS, Sigma), and resuspended in cold lysis buffer. The cells were placed on ice for 20 min, and lysed cells were centrifuged at 14,000*g* for 15 min. For the caspase assay, samples in assay buffer were mixed with caspase substrate (Ac-DEVD-pNA) in a 96-well plate. After overnight incubation at 37 °C, the absorbance of released *p*-nitroaniline was measured at 405 nm using a microplate reader (Bio-Rad Model 3550, Bio-Rad, Hercules, CA, USA). The caspase assay was performed in three replicates.

Macrophage stimulation and measurement of proinflammatory mediator and cytokine release

The murine macrophage cell line J774 was cultured in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Gibco BRL, Gaithersburg, MD, USA) at 37 °C in an atmosphere of 5% CO_2. Cells were placed in 24-well plates (4×10^5 cells/well) for 24 h, and then recombinant AvMaSp-R (250 μg per ml of medium) or lipopolysaccharide (100 ng per ml of medium, LPS, Sigma) was added to each well. After incubation for 5 h, cells were centrifuged at 600*g* for 10 min, and media were harvested for measurement of proinflammatory mediator and cytokine release via western blot analysis. The medium samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was performed using an enhanced chemiluminescence Western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA) with mouse polyclonal antibodies, including anti-tumor necrosis factor (TNF)-α antibody [diluted 1:1000 (v/v); Cell Signaling Technology, Beverly, MA, USA], anti-cyclooxygenase (COX)-2 antibody [diluted 1:1000 (v/v); Cell Signaling Technology], anti-interleukin (IL)-1β antibody [diluted 1:1000 (v/v); Abcam, Cambridge, United Kingdom], and anti-IL-6 antibody [diluted 1:1000 (v/v); Cell Signaling Technology]. Horseradish peroxidase-conjugated anti-mouse IgG [diluted 1:5000 (v/v); Sigma] was used as a secondary antibody.

Results and discussion

Cytotoxicity of recombinant AvMaSp-R spider silk fibroin protein was evaluated in NIH 3T3 murine fibroblast cells. None of the concentrations of recombinant AvMaSp-R assayed in this study induced cytotoxicity compared to Triton X-100 as a positive control (Fig. 1A). The results

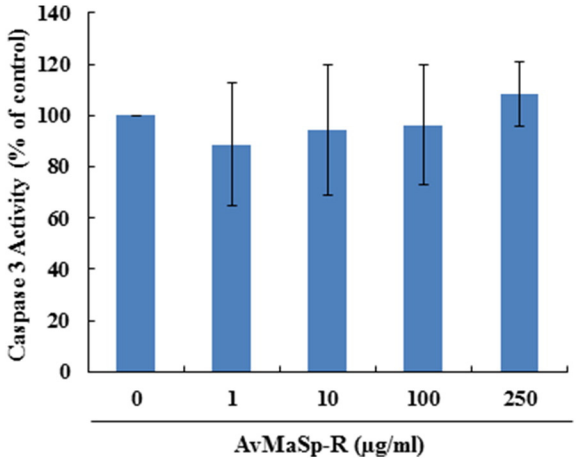

Fig. 2. Caspase 3 activity after treatment with recombinant AvMaSp-R. NIH 3T3 cells were treated with recombinant AvMaSp-R (0.1, 1, 10, 100, or 250 μg per ml of medium). Caspase 3 activity was determined at 24 h after treatment. Bars represent the mean ± SD (n = 3).