World Academy of Science, Engineering and Technology
International Journal of Medical and Health Sciences
Vol:4, No:5, 2010

Evaluation of the Immunoregulatory Activity of rFip-gts Purified from Baculovirus-infected Insect Cells

Tzong Yuan Wu, Sheng Kuo Hsieh, Tzyy Rong Jinn *

***Abstract*—**Fip-gts, an immunomodulatory protein purified from *Ganoderma tsugae*, has been reported to possess therapeutic effects in the treatment of cancer and autoimmune disease. For medicinal application, a recombinant Fip-gts was successfully expressed and purified in Sf21 insect cells by our previously work. It is important to evaluate the immunomodulatory activity of the rFip-gts. To assess the immunomodulatory potential of rFip-gts, the T lymphocytes of murine splenocytes were used in the present study. Results revealed that rFip-gts induced cellular aggregation formation. Additionally, the expression of IL-2 and IFN-r were up-regulated after the treatment of rFip-gts, and a corresponding increased production of IL-2 and IFN-r in a dose-dependent manner. The results showed that rFip-gts has an immunomodulatory activity in inducing Th1 lymphocytes from murine splenocytes released IL-2 and IFN-γ, thus suggest that rFip-gts may have therapeutic potential in vivo as an immune modulator.

***Keywords*—**Fungal immunomodulatory protein, *Ganoderma tsugae*, Interleukin 2, Interferon γ, Lingzhi

I. INTRODUCTION

Ganoderma tsugae (*G. tsugae*) is one of the most famous Lingzhi mushrooms, has been used to promote health and longevity in Asia countries [1]. Among potent bioactive components in *G. tsugae*, a glycoprotein has been identified which namely fungal immunomodulatory protein-gts (Fip-gts) [2]. As reported earlier, Amino acid sequences of Fip-gts and LZ-8 (a potent immunomodulatory protein purified from *Ganoderma lucidum*) were found identical. The mature LZ-8 polypeptide composed of 110 amino acid residues including an acetylated N-terminal serine was shown to share considerable similarity to the V_H region of immunoglobulins in both their primary sequences and predicted secondary structures [3-4]. Fip-gts can induce macrophages and T lymphocytes to produce interleukin 2 and tumor necrosis factor-α [5]. In addition, Fip-gts also can significantly inhibit the proliferation and invasion of tumor cells [6-8]. Thus, Fip-gts has the potential to use in the therapeutic treatment of tumors and immune diseases. For expression and production of the glycosylated Fip-gts in insect cells, a recombinant baculovirus $vAcP_{10}SP_{bbx}Gts$ was generated by co-transfection linearized AcRP23.LacZ DNA with plasmid $pAcP_{10}SP_{bbx}Gts$ DNA, encoding a bombyxin signal peptide sequence fused to a Fip-gts-6His sequence, in our previously study [9,10]. The recombinant Fip-gts (rFip-gts) was purified from the supernatant of the infected-Sf21 cells using a Ni-chelated affinity column, and was verified by Western blot and MALDI-MS analyses [10]. The yield of purified glycosylated rFip-gts was 166 μg from $1.1x10^9$ infected Sf21 insect cells in culture [10]. It is well known that Fip-gts possesses stimulatory activities toward human peripheral blood leucocytes. As expected, the purified 10μg/ml of glycosylated rFip-gts obviously enhanced the expression of IL-2, to a level of 1460 pg/ml [10]. For further evaluation the immunomodulatory activity of rFip-gts, which three different concentrations (1, 5 and 10 μg/ml) of rFip-gts were examined via the induction of interleukin 2 (IL-2) and interferon γ (IFN-γ) gene expression and measured IL-2 and IFN-γ released from murine splenocytes in the present study.

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II. MATERIALS AND METHODS

Purification of rFip-gts and glycoprotein staining

6His-tagged rFip-gts from recombinant baculoviruses $vAcP_{10}SP_{bbx}Gts$-infected Sf21 cell culture supernatant was purified as previously described [9]. All purification steps were carried out at 4 °C. Infected Sf21 cell culture supernatant containing rFip-gts were dialyzed by native binding buffer (50 mM NaPO4, 0.5 M NaCl, pH 8.0). The dialyzed supernatant was combined with 5 ml of 50% Ni-NTA slurry (Novagen, Darmstadt, Germany) in binding buffer and incubated with agitation overnight. The slurry was poured into a His-bind quick column and drained. The column was then washed with 10 volumes of lysis buffer and 6 volumes of wash buffer (500 mM NaCl, 20 mM Tris-HCl, and 60 mM imidazole, pH 7.9) and eluted with native elution buffer (binding buffer plus 250 mM imidazole). For glycoprotein staining, purified rFip-gts was resolved on a 15% SDS-PAGE gel and stained according to the description of the manufacturers of the Gelcode® glycoprotein staining kit (Pierce, USA).

Mouse splenocytes

The splenocytes of naïve mice (Balb/C; 8-10 weeks old) were isolated by the Ficoll-Hypaque method and resuspended to 1×10^7 cells/ml in RPMI 1640 medium (GibcoBRL,

Frederick, MD) supplemented with 10% FBS, 100 units/ml penicillin, 200 mmole/l L-glutamate, and 10 mmole/l Hepes, pH 7.3 [11]. Splenocytes were used to examine the immunoregulatory activity of rFip-gts as described below. Splenocytes were seeded in 0.1 ml of medium into the wells (1×10^6 cells/well) of a 96-well plate (Nunc, Rochester, NY), and the RPMI 1640 medium was added to obtain a final rFip-gts concentration of 1, 5 or 10 μg/ml. After incubation at 37 °C under 5% CO_2 for 48 h, were observed under a light microscope (Olympus type IX700).

Determination of IL-2 and IFN-γ in mouse splenocytes

The immunomodulatory activity of rFip-gts was examined via the induction of interleukin 2 (IL-2) and interferon γ (IFN-γ) gene expression and measured IL-2 and IFN-γ released from murine splenocytes. For total RNA isolation, the collected splenocytes were extracted according to the manufacturer's instructions (Perkin Elmer, USA) for first strand cDNA synthesis. RT-PCR was performed using specific oligonucleotide primers for the IL-2 (413-bp product), IFN-γ (460-bp product) and β-actin genes (566-bp product). RT-PCR products (20 μl) were separated on a 1% agarose gel. Densitometric analysis of PCR products was performed with an AlphaImager 2200 digital imaging system (Alpha Innotech, San Leandro, CA, USA). The levels of IL-2 in the culture supernatants were determined by ELISA using a commercial IL-2 kit (Biosource, Carlsbad, California). On the other hand, IFN-γ were determined by a commercial IFN-γ kit (Signosis, Sunnyvale, CA, USA) as described by the manufacturers.

III. Results and Dicussion

The glycosylated rFip-gts was purified from the recombinant baculoviruses $vAcP_{10}SP_{bbx}$Gts-infected Sf21 cells culture medium by nickel-chelated affinity chromatography and was analyzed by glycoprotein staining. As revealed in Fig. 1, the purified rFip-gts protein of ~15 kDa was found to be glycosylated.

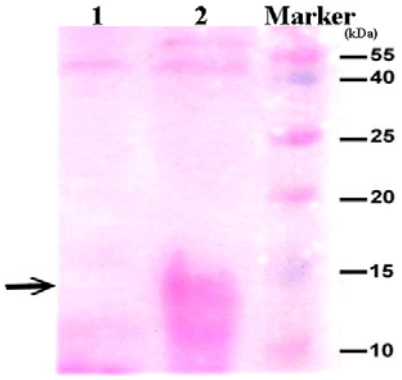

Fig. 1. Detection of affinity purified rFip-gts. rFip-gts was purified by nickel-chelated affinity chromatography under native conditions. The purity of rFip-gts was resolved on a 15% SDS-PAGE gel and then was stained by a glycoprotein staining kit. Lane 1, uninfected Sf21 cells; Lane 2, $vAcP_{10}SP_{bbx}$Gts -infected Sf21 cells. The positions of the rFip-gts proteins are represented by the solid arrows.

To better assess the immunomodulatory potential of rFip-gts, the T lymphocytes of murine splenocytes were isolated and cultured in this study. It was observed by microscopy that the T lymphocytes of murine splenocytes treated with rFip-gts formed obvious cellular aggregates, whereas untreated splenocytes remained in a single cell adhesion (Fig.2). In additional, the formation of cell aggregates showed a dose-dependent manner (Fig.2). As reported, the hPBL cellular aggregates are coincident with the upregulation of ICAM-1 expression [12].

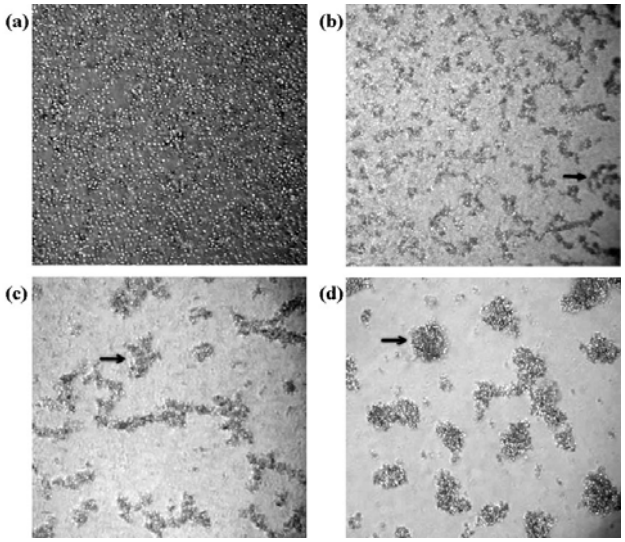

Fig. 2. Mouse splenocytes aggregate formation in response to different concentrations of glycosylated rFip-gts. Mouse splenocytes were incubated with PBS (a) and one of three concentrations of rFip-gts for 1 μg/ml (b), 5 μg/ml (c), and 10 μg/ml (d) at 48h, and then splenocytes were observed under a light microscope (magnification 40X). The positions of cells aggregate formation are represented by the solid arrows.

Moreover, we examined whether rFip-gts could regulate IL-2 gene expression in murine splenocytes using a semi-quantitative RT-PCR method. In this treatment, mouse β-actin was used as a control amplicon to assess constitutive transcription rates. As shown in Fig. 3(a), the rFip-gts could significantly enhance the transcriptional level of IL-2 cytokine by a dose-dependent effect in murine splenocytes. To further confirm the induction of IL-2 cytokine release in murine splenocytes, an IL-2 cytokine ELISA assay system was employed and PBS as a negative control. The detected IL-2 levels were 530, 1006 and 1670 pg/ml while splenocytes were treated with 1, 5 and 10 μg/ml of rFip-gts, respectively. The data provide evidence for a dose-dependent effect on rFip-gts inducing the release of IL-2 from splenocytes (Fig. 3b). The results significantly demonstrate that the rFip-gts produced from $vAcP_{10}SP_{bbx}$Gts-infected insect cell possess the highly potency in inducing the release of IL-2 from splenocytes.

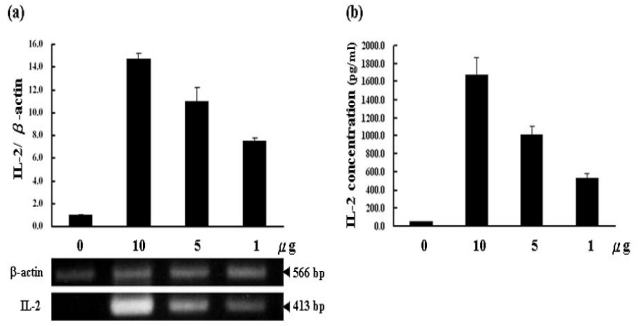

Fig. 3. Effects of the immunomodulatory activity of rFip-gts on the release of IL-2 cytokine from murine splenocytes. The expression of the IL-2 gene and the release of IL-2 cytokine in murine splenocytes were examined by RT-PCR and the ELISA method. Densitometric quantification revealed the level of gene expression of the IL-2 cytokine and β-actin (a). The release of IL-2 cytokine from murine splenocytes was then examined by ELISA readings under 540 nm with reference to an internal control (b). These data were obtained from three separate experiments and are presented as the mean ± standard deviation.

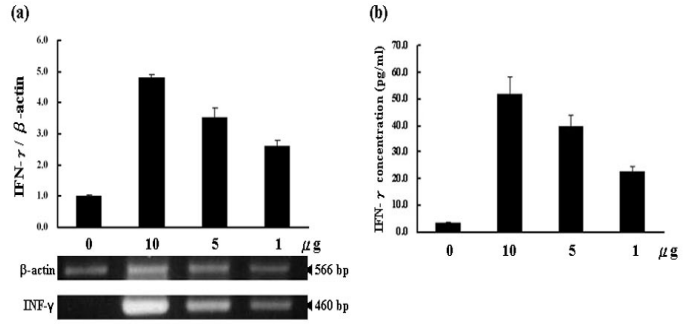

Fig. 4. Effects of the immunomodulatory activity of rFip-gts on the release of IFN-γ from murine splenocytes. The expression of the IFN-γ gene and the release of IFN-γ cytokine in murine splenocytes were examined by RT-PCR and the ELISA method. Densitometric quantification revealed the level of gene expression of the IFN-γ and β-actin (a). The release of IFN-γ from murine splenocytes was then examined by ELISA readings with reference to an internal control (b). These data were obtained from three separate experiments and are presented as the mean ± standard deviation.

Meanwhile, we also examined whether rFip-gts could regulate IFN-γ gene expression in murine splenocytes. As shown in Fig. 4a, rFip-gts possesses the capable of causing the upregulation of IFN-γ gene expression. To confirm the induction of IFN-γ cytokine release in murine splenocytes, an IFN-γ cytokine ELISA assay system was employed. The detected IFN-γ levels were 52, 40 and 23 pg/ml while splenocytes treated with 1, 5 and 10 μg/ml of rFip-gts, respectively (Fig. 4b). The results showed that rFip-gts has an immunomodulatory activity in inducing Th1 lymphocytes from murine splenocytes released IL-2 and IFN-γ. These data suggest that rFip-gts may have therapeutic potential in vivo as an immune modulator.

ACKNOWLEDGMENT

The authors would like to thank the China Medical University and National Science Council of the Republic of China for financially supporting this research under Contract No. CMU98-N1-23 and NSC-98-2324-B-039-005.

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