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Oral administration of an Enoki mushroom protein FVE activates innate and adaptive immunity and induces anti-tumor activity against murine hepatocellular carcinoma

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ABSTRACT

FVE is a documented immunomodulatory protein purified from Enoki mushroom (*Flammulina velutipes*) and known as an activator for human T lymphocytes. This present study was aimed to investigate the anti-tumor effect and the related mechanisms of oral administration of FVE using a murine hepatoma model. Oral administration of FVE (10 mg/kg) significantly increased the life span and inhibited the tumor size of BNL 1MEA.7R.1 (BNL) hepatoma-bearing mice. Tumor-bearing mice receiving oral FVE treatment had the highest tumoricidal capacity of peritoneal macrophages and tumor-specific splenocytes against BNL hepatoma cells. In addition, in vivo neutralization of interferon-gamma (IFN-γ) demonstrated a significant decrease of FVE-induced anti-tumor effect ($P<0.05$). The expression levels of major histocompatibility complex (MHC) class I and II molecules and costimulatory molecule CD80 on peripheral blood mononuclear cells obtained from the FVE-treated mice were upregulated as compared with those of the PBS-treated mice. Furthermore, immunohistochemical staining showed a strong inhibition of tumor growth and angiogenesis in hepatoma tissues after oral administration of FVE. Taken together, oral administration of FVE displayed anti-tumor activity through activating both innate and adaptive immunity of the host to prime a cytotoxic immune response and IFN-γ played a key role in the anti-tumor efficacy of FVE.

1. Introduction

Edible mushrooms have an established history of use in the Orient as a highly nutritious foodstuff. Based on its high nutrition value, some mushroom subspecies have been used in medicinal purposes and have displayed profound health-promoting benefits [1]. Recently, the ability of mushrooms on tumor prevention and therapeutic usage against tumor growth has been demonstrated in both in vitro and in vivo model systems [2]. Some polysaccharide and protein complexes from mushrooms were able to enhance the non-specific immune responses and to exert anti-tumor activity through the stimulation of the host's defense mechanism [3]. Besides the strong scientific evidence for anti-tumor activity from medicinal mushrooms in laboratory studies, many new anti-tumor compounds have been identified and put into practical use. Previous studies showed that many medicinal applications come from the commercial compounds which were isolated from Basidiomycetes class of fungi, such as lentinan, PSK (krestin), and schizophyllan [4]. There were also some impressive new data for relatively pure bioactive compounds from *Grifola frondosa* (maitake), *F. velutipes*, *Pleurotus ostreatu* and others [5].

FVE, a well-characterized immunomodulatory protein isolated from the edible mushroom *F. velutipes*, showed the stimulatory activity toward human peripheral blood lymphocytes [6]. It was classified to a distinct family of fungal immunomodulatory proteins (FIPs) that exhibited amino acid sequence and biological activity similarity [7]. The FVE was a 12.7 kDa glycoprotein consisting of 114 amino acid residues, and its cDNA has been cloned and sequenced [8]. The FVE was shown to be capable of activating peripheral blood mononuclear cells with a concomitant increase in interferon-gamma (IFN-γ) production through p38 mitogen-activated protein kinase signaling pathway [9]. In addition, oral administration of FVE could induce a Th1-predominant allergen-specific immune response to efficiently suppress systemic anaphylaxis-like symptoms [10]. It was also reported that the immunoprophylatic effects of FVE in Th2-mediated allergic anaphylaxis were associated with the ability of FVE to enhance activation of IFN-γ-releasing Th1 cells and inhibit IL-5-mediated survival of eosinophils [9,11]. IFN-γ was believed to play a key role in FVE-mediated immune response, leading to subsequent priming of the Th1 differentiation [9–11].

IFN-γ is a pleiotropic cytokine produced mainly by activated T lymphocytes and natural killer cells. It is well known that IFN-γ is capable of triggering multiple cellular responses including antiviral

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activity, inhibition of cell growth, modulation of cell differentiation and immunoregulation [12]. Current attention is focused on the potential anti-tumor effects of IFN-γ, which exerts its anti-tumor actions via either direct anti-proliferative effects on tumor cells or indirectly through the host immune system, including enhancement of major histocompatibility complex (MHC) class I and II expression, activation of macrophage and natural killer cells, generation of cytotoxic T lymphocytes and induction of tumor associated antigen [13]. Because of these biological activities, IFN-γ has been used as an anti-tumor agent and tested in clinical trials as a therapeutic for treating malignant tumors [14,15]. As occurred with the clinical evolution of combination chemotherapy, recent studies have demonstrated that IFN-γ with less side effects could be utilized together to achieve a maximum therapeutic benefit for cancer patients. For these reasons, IFN-γ is a pivotal cytokine involved with the activation of innate and adaptive anti-tumor immune responses.

In contrast to the medicinal use for immune regulation and allergy treatment, very little data from clinical applications in anti-tumor immune therapy have been generated using FVE modulation. Accordingly, the aim of this study was to evaluate immune responses particularly referred to the in vivo anti-tumor effect of FVE via oral administration on hepatic cancer in mice. Based on our previous findings, we further investigated whether the neutralization of IFN-γ during FVE administration could cause a reduction in tumor suppression, then to identify the role of IFN-γ in triggering immune cells to attack tumor cells induced by oral administration of FVE. These studies were able to provide us with useful information on the improved efficacy of complementary FVE therapy on malignant tumors.

2. Materials and methods

2.1. Preparation of FVE

FVE was purified following a previously described method [6] with minor modifications and all purification steps were carried out at 4 °C. Fresh fruiting bodies of the golden needle mushroom (*F. velutipes*) were homogenized with a cold 5% (v/v) acetic acid solution in the presence of 0.1% (v/v) 2-mercaptoethanol. The homogenates were centrifuged at 8500 *g* for 40 min and soluble proteins in the supernatant were precipitated by addition of ammonium sulfate up to 95% of saturation with stirring overnight. The precipitates were collected by centrifugation at 10,000 *g* for 1 h and then dialyzed against 10 mM sodium acetate (pH 5.2) for 72 h with four changes of dialysis solution. The dialysate was applied to a CM-52 cellulose column (2.5 cm × 20 cm; Whatman, Maidstone, UK) that was previously equilibrated using 10 mM sodium acetate at pH 5.2. The column was washing with 500 ml equilibration buffer to remove unbound proteins and then eluted with 300 ml of 0–0.5 M NaCl in 10 mM sodium acetate (pH 5.2). The main fractions were further purified by a fast protein liquid chromatography (FPLC) system with a Mono S HR 5/5 column (Amersham Biosciences, Uppsala, Sweden) previously equilibrated with 10 mM sodium acetate (pH 5.2). The column was first washed with 50 ml equilibration buffer and then eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. Fractions of FVE as a single peak on absorbance at 280 nm were collected and dialyzed against phosphate-buffered saline (PBS) for oral administration. The protein was present as a single band on SDS-PAGE at 12.7 kDa (Supplementary Fig. S1). The activity of FVE was checked with a proliferation assay on mouse splenocytes. Furthermore, we excluded the possibility of endotoxin contamination of FVE by the *Limulus* amoebocyte lysate (LAL) clot assay (Supplementary Table S1).

2.2. Cell lines and animals

The murine BNL 1MEA.7R.1 (BNL) hepatoma cell line derived from BALB/c mice was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY, USA), and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Female BALB/c mice aged between 6 and 8 weeks obtained from the Animal Center of National Taiwan University (Taipei, Taiwan) were maintained in the animal facility under temperature-controlled conditions. All animal studied were carried out according to the Institutional Animal Care and Use Committee of National Taiwan University (permission number NTU-93-040).

2.3. Preparation of anti-IFN-γ monoclonal antibodies

Rat anti-mouse IFN-γ monoclonal antibody R4-6A2 (ATCC HB-170) used for in vivo injection to neutralize IFN-γ activity in mice was purified from hybridoma cell culture supernatants following ammonium sulfate precipitation and affinity chromatography on a protein G-sepharose column (HiTrap; Amersham Biosciences). Purified anti-IFN-γ monoclonal antibodies were dialyzed against PBS and concentrated by using an Amicon concentrator (Millipore, Billerica, MA, USA) with a 10 kDa-molecular-mass-cutoff membrane.

2.4. Oral administration of FVE

Mice were divided into two groups of which each was consisted of twelve mice. A group was administered by gavage 0.2 ml of FVE solution (1 mg/ml in PBS) at a dose of 10 mg/kg body weight (200 μg per mouse). Another group served as the normal control received an equal volume of PBS. All groups were injected intraperitoneally (i.p.) with 5×10^4 viable BNL hepatoma cells in 500 μl of PBS per mouse prior to oral administration. FVE and PBS were administered four times a week (every other day) at weekly intervals, as shown in Fig. 1.

To further investigate whether IFN-γ was involved in anti-tumor activity of FVE, each oral treatment was subdivided into two groups that were injected intraperitoneally with or without 25 μg of anti-IFN-γ antibodies (R4-6A2) in 100 ml PBS once every two weeks. The tumor size was recorded by measuring the length, width and height of each tumor once every two weeks. The survival time of tumor-bearing mice was also monitored daily until death after tumor inoculation.

2.5. Determination of tumoricidal activity

BNL hepatoma cells were pretreated with 50 μg/ml of mitomycin C (Sigma, St. Louis, MO, USA) to inhibit cell proliferation for 24 h. After extensive washing five times with PBS to remove excess mitomycin C, the pretreated tumor cells were used as target cells. Hepatoma-bearing mice with intraperitoneal injection of anti-IFN-γ antibodies or rat IgG isotype control (BD Pharmingen, San Diego, CA, USA) were orally administered with either FVE (200 μg per mouse) or PBS everyday at weekly intervals for a month. After oral administration, tumor-bearing mice were sacrificed to obtain splenocytes suspended in DMEM supplemented with 10% (v/v) FBS. Then, splenocytes were activated with BNL hepatoma cells pretreated with mitomycin C for 3 days. The activated splenocytes were used as effector cells. Target cells were cultured in 96-well plates (10^3 cells/well) at 37 °C in air with 5% CO_2 overnight. After culturing overnight, 5-bromo-2′-deoxyuridine (BrdU; Roche Diagnostics, Mannheim, Germany) was added at a final concentration of 10 μM to each well for 4 h. Then the culture medium in each well was removed and washed with PBS three times. According to effector-to-target cell ratios of 10:1, 30:1, 100:1, and 300:1, target cells (10^3 cells/well) were incubated with different amounts of effector cells (10^4, 3×10^4, 10^5, 3×10^5 cells/well, respectively) for 4 h. The tumoricidal activity of splenocytes (effector cells) was measured by using the BrdU cell proliferation assay (Roche) according to the manufacturer's instructions. Cell survival curves (serial dilution of 5000 cells/well) were generated according to the levels of BrdU incorporation in known amounts of target cells. The results are