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Anti-angiogenic effects and mechanisms of polysaccharides from *Antrodia cinnamomea* with different molecular weights

Chih-Min Yang[a], Yu-Jie Zhou[a], Ran-Juh Wang[b], Miao-Lin Hu[a,*]

[a] *Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 402, Taiwan, ROC*
[b] *Miaoli District Agricultural Research and Extension Station, Council of Agriculture, 261 Guannan Tsuen, Gungguan Shiang, Miaoli 363, Taiwan, ROC*

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ABSTRACT

Ethnopharmacological relevance: Antrodia cinnamomea is a popular medicinal mushroom in Taiwan that has been widely used for treatment of various cancers and liver diseases.
Aim of the study: This study aimed to investigate the immunomodulatory effect on angiogenesis of polysaccharides from mycelia of *Antrodia cinnamomea* (PMAC).
Materials and methods: PMAC were extracted in boiling water, precipitated with 95% ethanol, and separated into four different molecular weights (<5, 5–30, 30–100, >100 kDa). Tube formation and chorioallantoic membrane (CAM) assay were used to determine the *in vitro* and *ex vivo* anti-angiogenic effects.
Results: Only the PMAC-mononuclear cells (MNCs)-conditioned medium (CM) with MW > 100 kDa significantly and concentration-dependently decreased the secretion of vascular endothelial growth factor in human leukemia cells and inhibited the matrigel tube formation in human umbilical vein endothelial cells. Similarly only the PMAC-MNC-CM with MW > 100 kDa significantly and concentration-dependently increased the levels of interleukin (IL)-12 and interferon-γ (IFN-γ). In addition, the *ex vivo* CAM assay revealed that only the PMAC with MW > 100 kDa significantly and dose-dependently inhibited neovascularization.
Conclusions: PMAC with MW > 100 kDa are anti-angiogenic *in vitro* and *ex vivo*, and the effects are likely through immunomodulation.

1. Introduction

Angiogenesis, the formation of new blood vessels from preexisting ones, plays a key role in tumor growth and metastasis (Folkman, 1990). Angiogenesis requires endothelial proliferation, migration, and tube formation. Several specific factors are known to regulate angiogenesis, including vascular endothelial growth factor (VEGF), interleukin (IL)-8, IL-12 and others (Oppenheim and Fujiwara, 1996; Rodolfo and Colombo, 1999; Ferrara, 2000). Inhibition of angiogenesis is a promising strategy for the treatment of cancer (Ferrara and Kerbel, 2005).

Mushrooms have been used as food and medicine by humans for centuries (Wasser, 2002). Polysaccharides isolated from mushrooms are the most promising compounds because of their immunomodulatory and antitumor effects (Chen et al., 2004) and have been shown to inhibit the proliferation of cancerous cells indirectly by activating the host immune responses (Borchers et al., 2004). The antitumor activities of polysaccharides isolated from mushrooms are most attractive due to their low toxicity to normal cells and the apparent lack of side effects in clinical patients (Fukushima, 1989). For examples, lentinan from *Lentinus edodes*, krestin (PSK) from *Trametes versicolor*, and schizophyllan from *Schizophyllum commune* have been used for clinical cancer immunotherapy in Japan (Wasser and Weis, 1999; Ikekawa, 2001).

Antrodia cinnamomea (AC) also called "niu-chan-ku" or "chang-chih", is a species known to be available only in Taiwan. It was initially identified by Zang and Su (1990) as new *Ganoderma* species. Many investigations have reported the biological activities of triterpene acids, steroid acids and polysaccharides isolated from *Antrodia cinnamomea* (Chen et al., 1995; Nakamura et al., 2004; Lu et al., 2008). Polysaccharides from mycelia of *Antrodia cinnamomea* (PMAC) have been shown to possess activity against anti-hepatitis B surface antigen (Lee et al., 2002) and to directly suppress angiogenesis of endothelial cell *in vitro* by inhibition of VEGF receptor signaling (Cheng et al., 2005). By contrast, PMAC were found to

Abbreviations: CAM, chorioallantoic membrane; CM, conditioned medium; HL-60, human leukemia cells; HUVECs, human umbilical vein endothelial cells; IFN-γ, interferon-γ; IL-12, interleukin-12; MNC, mononuclear cells; PMAC, polysaccharides from mycelia of *Antrodia cinnamomea* (PMAC); VEGF, vascular endothelial growth factor.

* Corresponding author. Tel.: +886 4 2281 2363; fax: +886 4 2281 2363.
E-mail address: nlhuhu@dragon.nchu.edu.tw (M.-L. Hu).

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inhibit the proliferation of human leukemia U937 cells through the activation of host immune response both *in vitro* and *in vivo*, rather than by direct cytotoxicity against U937 cells (Liu et al., 2004). Indeed, it has been concluded that polysaccharides from mushroom do not attack cancerous cells directly, but produce their antitumor effects by activation of immune response in the host (Wasser, 2002). Nevertheless, few reports have investigated the relationship between PMAC with different MW fraction and the anti-angiogenic activity. In this study, we investigated the anti-angiogenic effects of various molecular weight fraction (<5, 5–30, 30–100, >100 kDa) of PMAC *in vitro*, and we hypothesized that the main anti-angiogenic activity is associated with higher MW fraction (i.e. >100 kDa) and that the effect is through immunomodulation.

2. Materials and methods

2.1. Materials

Human promyelocytic leukemia cell line (HL-60, BCRC 60027) and human umbilical vein endothelial cell line (HUVEC, BCRC H-UV001) was purchased from Food Industry Research and Development Institute, Hsin Chu, Taiwan. Phytohemagglutinin (PHA) was purchased from Sigma (St. Louis, MO). Ficoll-Hypaque/GE was purchased from Healthcare (Sweden).

2.2. Extraction and fractionation of PMAC

Mycelia of *Antrodia cinnamomea* were obtained from the Biotechnology Center, Grape King Inc., Chungli, Taiwan. The voncher specimen (BCRC 35396) is deposited in the Food Industry Research and Development Institute, Hsin Chu, Taiwan. The polysaccharide fraction of mycelia of *Antrodia cinnamomea* (PMAC) was isolated according to a published method (Liu et al., 2004) with minor modification. Briefly, the mycelia of *Antrodia cinnamomea* were air-dried and extracted with boiling water (at a ratio of 1:20, w/v) for 6 h. The suspension was filtered under suction to remove the insoluble matter. The filtrate was mixed with four volumes of ethanol (95%) and allowed to stand overnight to precipitate crude polysaccharides. After centrifugation ($8 \times g$, 20 min) to remove the supernatant, the crude polysaccharides were dissolved in small amounts of water. The crude PMAC were then separated into four molecular weight fractions (<5, 5–30, 30–100 and >100 kDa) by centrifugation using Amicon Ultra (Millipore, Ireland) followed by lyophilization (KINGMECH FD50L-8S, Taiwan). The PMAC with MW > 100 kDa were further analyzed using high-pressure liquid chromatograms (HPLC) (Waters^{TM} 600 controller) with Waters 410 Differential Refractometer and Water 717_{plus} Autosampler. Samples (20 μl) were injected onto a POLYSEPTM-GFCP4000 column, and the column was eluted with ddH_2O at a flow rate of 0.8 ml/min. The molecular weights were confirmed using dextran standards (5, 50, 80, 150, and 410 kDa) (Sigma–Aldrich, Buchs, Schweiz) by the retention time.

2.3. Assay of polysaccharide, β-1, 3-glucan contents and neutral sugars composition in PMAC

The polysaccharide content of lyophilized PMAC was measured by the phenol–sulfuric acid colorimetric method (Dubois et al., 1956). Briefly, PMAC dissolved in 20 volumes of ddH_2O was mixed evenly with phenol and then sulfuric acid. The polysaccharide content of the mixture was determined spectrophotometrically at 490 nm by using a standard curve of glucose solution. To determine β-1, 3-glucan content (Ko and Lin, 2004), PMAC were dissolved in ddH_2O followed by addition of 2.5 ml of 0.3N NaOH. After titrating the pH to 11.5 by HCl, the volume of the mixture was adjusted to 10 ml with Na_2HPO_4–NaOH buffer. Aniline blue (0.2 ml 1.0 mg/ml) was added to 2 ml of the mixture and allowed to stand for 2 h. The fluorescence intensity (at an excitation of 395 nm and an emission of 495 nm) was quantified by a standard curve of activated polysaccharide (Laminarin).

The neutral sugars composition in the four MW fractions of PMAC were measured according to published methods (Hardy et al., 1988) with minor modification. A portion (100 mg) of the PMAC was added to 5 ml of 2.0 M trifluoroacetic acid and hydrolyzed at 95 °C for 16 h. The neutral sugar composition was measured using HPLC (Shimadzu LC-10 AT *VP*) with Shimadzu RID-10A detector. The column was eluted with acetonitrile and ddH_2O (85:15, v/v) at a flow rate of 1 ml/min.

2.4. Preparation of conditioned medium

Human peripheral blood was obtained from four normal adult volunteers (aged between 24 and 28) with informed consent, and mononuclear cells (MNCs) of each person were separated by density centrifugation ($400 \times g$, 30 min) in Ficoll-Hypaque solution (1.077 g/ml) (Ou et al., 2005). MNCs recovered at the interface were washed twice in Hank's balanced salt solutions (HBSS) and resuspended in RPMI 1640 medium containing 10% FBS. To obtain conditioned media (CM), MNCs (1×10^6 cells/ml) were incubated with RPMI 1640 medium containing 10% FBS with or without PMAC at 37 °C for 3 days, after which the CM were collected, filtered (0.45 μm), and stored at −70 °C until use. For convenience, these PMAC-stimulated-MNC-CM were named PMAC-MNC-CM. As a positive control, phytohemagglutinin (PHA) was used to prepare PHA-MNC-CM (Liu et al., 2004).

2.5. Cell culture

HUVECs were cultured in M199 medium (Gibco, USA), supplemented with 10% fetal bovine serum (FBS), 30 μg/ml endothelial cell growth supplement (ECGS), 25 U/ml heparin, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate and 1% antibiotic–antimycotic (Gibco, USA). HUVECs were seeded onto 10-cm dish coating 1% gelatin and incubated at 37 °C in a humid atmosphere containing 5% CO_2. HL-60 cells were cultured in PRMI (Gibco, USA) supplement with 10% fetal bovine serum (FBS), 0.37% (w/v) $NaHCO_3$, 100 units/ml penicillin and 100 μg/ml streptomycin. HUVECs and HL-60 were cultured at an initial concentration of 1×10^5 and 1×10^6 cells/ml in the presence of 30% (v/v) PMAC-stimulated-MNC-CM.

2.6. Assay of tube formation in HUVECs

The effect of PMAC on angiogenesis *in vitro* was estimated by the tube formation assay, as described previously (Cheng et al., 2005). Briefly, HUVECs (1×10^5 cells/ml) were cultured into 96-well culture plates, which were pre-coated with the 50 μl/well ECMatrix (Chemicon, USA). Cells were treated with PMAC-MNC-CM or non-stimulated-MNC-CM for 6, 12 and 24 h, and then the tube formation was examined by microscopy. For each replicate, the cells in 10 randomly selected fields were determined.

2.7. Assay of cytokines in MNC-CM and vascular endothelial growth factor secreted by HL-60 cells

Cytokines including interleukin (IL)-12, IFN-γ, IL-8 and PGE2 in MNC-CM were quantified using commercial kits with a solid-phase ELISA (R&D systems, Inc., USA) at a wavelength of 450 nm (Liu et al., 2004). MNC-CM was diluted 1–5-fold with a commercial dilution solution before assay. VEGF in the medium of HL-60 cells was determined using ELISA Kit (Endogen Human ELISA Kit,