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Effect of polymeric caffeic acid on antitumour activity and natural killer cell activity in mice

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ABSTRACT

The antitumour activity and immunomodulating activity of synthesized lignin-like polyphenols in mice were determined. Polymerized polyphenols were prepared using caffeic acid as the precursor. The intake of both polymeric and monomeric caffeic acid inhibited the growth of B7-P815 tumours in mice, but we found that only the monomers inhibited tumour growth *in vitro*. Oral administration of polymerized caffeic acid, but not monomeric caffeic acid, substantially increased interferon-γ production in splenocytes stimulated with phorbol 12-myristate 13-acetate and ionomycin. Moreover, natural killer activity was markedly upregulated in mice that were administered polymerized caffeic acid compared with mice administered water or caffeic acid monomers. Lastly, we determined that caffeic acid monomers were absorbed more easily than polymers, but ingested polymers were less susceptible to degradation into monomers by digesting enzymes. Taken together, our results suggest that polymerized caffeic acid administered orally can upregulate systemic immunity and inhibit tumour cell growth.

1. Introduction

Lignin is a naturally occurring high-molecular-weight phenolic compound in plants. Natural lignins have been demonstrated to be beneficial for human health because they possess properties such as anti-influenza virus (Sakagami et al., 1992), anti-HIV (Lai et al., 1990), and anti-herpes simplex virus (Fukuchi et al., 1989) activities. In addition, many phenolic compounds have antioxidant activity that protects the intestinal tract (Carrasco-Pozo, Speisky, Brunser, Pastene, & Gotteland, 2011) and blood vessels (Stoclet et al., 2004) and inhibits carcinogenesis (Sequetto et al., 2013). Moreover, phenolic compounds derived from functional foods such as green tea leaf (Hirao et al., 2010), blueberry polyphenols (Lau, Joseph, McDonald, & Kalt, 2009), mulberry leaf (Park, Lee, Lee, & Kim, 2013), and pomegranate fruit (Rasheed et al., 2009) have been reported to modulate immune systems through suppression of mitogen-activated protein kinases and nuclear factor-κB activation. Although several functions of phenolic compounds have been uncovered, the effects of high-molecular-weight polyphenols on the immunological system remain poorly understood. Immunological studies with natural products are hindered by the contamination of the products, and natural lignins exist in plant tissues complexed with cellulose and hemicellulose. Therefore, previously we synthesized lignin-like polymerized polyphenols by using 3 types of hydroxycinnamic acids, caffeic acid (CA), *trans*-ferulic acid, and *trans*-*p*-coumaric acid, and demonstrated that these polyphenols induce cytokines in murine splenocytes (Yamanaka et al., 2012a). This suggested the

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possibility that polymerized polyphenols possess an immunoenhancing effect and may serve as food ingredients. However, the immunopharmacological actions of orally administered polyphenols have not been characterized in depth.

Numerous food-related functional molecules have a variety of immune-modulating abilities. For instance, β-glucans purified from edible mushrooms, which are used currently as complementary and alternative medicines for cancer immunotherapy, have been shown to upregulate the activity of natural killer (NK) cells and induce interferon (IFN)-γ synthesis (Liu et al., 2008; Yamanaka, Liu, Motoi, & Ohno, 2013a; Yamanaka et al., 2012b, 2012c). IFN-γ is a key molecule for food-mediated immunoactivation that functions in anticancer therapy and infection control because IFN-γ can upregulate the activity of NK cells and cytotoxic T lymphocytes (de Graaf, Horak, & Bookman, 1988; Djeu et al., 1982; Gidlund, Orn, Wigzell, Senik, & Gresser, 1978; Weigent, Stanton, & Johnson, 1983), and we have previously demonstrated that IFN-γ expression is induced by lignin-like polymerized polyphenols *in vitro* (Yamanaka et al., 2013b). These findings raised the possibility of a new application for lignin-like phenolic compounds in the treatment of cancer or infectious diseases. Therefore, to facilitate the use of functional food ingredients as alternative medicine, we investigated whether orally administered polymerized polyphenols demonstrate immunomodulating and antitumour activities.

In this study, we prepared polymerized polyphenols from the precursor CA through catalysis using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) and examined the antitumour effect of oral administration of polymerized caffeic acid (pCA).

2. Materials and methods

2.1. Animals and materials

Male C3H/HeN and female DBA/2 mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in a specific pathogen-free environment and were used when 4–8 weeks old. All animal experiments were performed in accordance with the guidelines for laboratory animal experiments provided by the Tokyo University of Pharmacy and Life Sciences, and each experimental protocol was approved by the Committee for Laboratory Animal Experiments at Tokyo University of Pharmacy and Life Sciences (P12–42, P13–45, P13–46). We purchased 3,4-dihydroxycinnamic acid (CA) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Calbiochem Inc. (San Diego, CA, USA), and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) from Wako Pure Chemical Co. (Osaka, Japan). Type II HRP, phorbol 12-myristate 13-acetate (PMA), and ionomycin calcium salt from *Streptomyces conglobatus* were purchased from Sigma (St. Louis, MO, USA), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Dojindo (Kumamoto, Japan). Anti-mouse CD16/CD32 (2.4G2) (Fc Block), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (RM4-5) (Rat IgG2a), and FITC-conjugated rat IgG2a isotype control were purchased from BD Biosciences (San Diego, CA, USA). Phyco-erythrin (PE)-conjugated anti-mouse CD80 (RMMP-1) (Rat IgG2a) was obtained from Caltag Laboratories (San Francisco, CA, USA), and PE-conjugated anti-mouse NKp46 (29A1.4) (Rat IgG2a), allophycocyanine (APC)-conjugated rat IgG2b isotype control, and PE-conjugated rat IgG2a isotype control were purchased from BioLegend (San Diego, CA, USA). APC-conjugated anti-mouse CD3e (17A2) (Rat IgG2b) and PE-conjugated anti-mouse CD8a (53-6.7) (Rat IgG2a) were purchased from eBioscience (San Diego, CA, USA).

2.2. Preparation of polymerized polyphenols

We performed enzymatic synthesis of lignin-like pCA by using HRP and CA as the enzyme and precursor, respectively, as described previously (Yamanaka et al., 2012a). Briefly, CA (200 mg) was neutralized using 1 M NaOH and diluted to 10 mL with phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4{\cdot}12H_2O$ in 1000 mL deionized water, pH 7.4) containing HRP (1 mg). H_2O_2 solution (30%) was diluted to 0.1% with PBS, and 1.5 mol eq of H_2O_2 was added dropwise to the mixture of precursor CA and HRP and stirred for 1 h at room temperature. The reaction mixture was stirred for 2 h more at room temperature and then heated for 20 min at 100 °C to inactivate HRP. After centrifugation, the supernatant was collected and dialyzed (MWCO: 50,000 Da) against deionized water for 2 d and then lyophilized. The yield based on precursor weight was 40.0% and both endotoxin (231.5 pg/mg) and protein (nitrogen: 0.02%) contamination in pCA were very low (Yamanaka et al., 2012a).

2.3. Gel-filtration chromatography

CA or pCA (1 mg) dissolved in 200 μL of acetone/8 M aqueous urea solution. (6:4, v/v) was applied to a Toyopearl HW-60F column (Tosoh Co., Ltd., Tokyo, Japan) (1 × 45 cm) equilibrated with acetone/8 M urea and fractionated. Eluted fractions (500 μL/tube) were collected and monitored using Folin–Ciocalteu reagent (Folin & Ciocalteu, 1927). Briefly, equal volumes of fraction samples and Folin–Ciocalteu phenol reagent (Sigma) were mixed at room temperature for 3 min and then 10% (w/v) sodium bicarbonate was added. After incubating at room temperature for 60 min, the absorbance at 700 nm was measured using a Safire microplate reader (TECAN, Salzburg, Austria).

2.4. Umu assay

The mutagenic activity of CA monomers and polymers against bacteria was determined using the *Umulac* AT kit (Protein Purify. Co. Ltd., Gunma, Japan) according to the manufacturer's instructions. Briefly, CA samples were dissolved in water for concentrations ranging from 15 ng/mL to 1 mg/mL; for concentrations over 2 mg/mL, the samples mixed thoroughly in 10% dimethyl sulphoxide (DMSO) (Sigma), with 10% DMSO serving as a control. These samples (10 μL each) were mixed with 100 μL of *Salmonella enterica* serovar Typhimurium strain NM2009 in the presence or absence of S9mix. After mixing on a shaking incubator at 37 °C for 2 h, induction of the *umu*