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Rhei Rhizoma Extracts Have Antiproliferative Properties and Differential Effects on NO Production in Macrophages

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Abstract

Recently, Rhei Rhizoma extracts (RRE) have begun to receive more attention as potential biological response modifiers. In the present study, we studied the antiproliferative effect of RRE on tumor cells and the effect of RRE on macrophage function. A variety of tumor cells and macrophages were treated with RRE at various concentrations. The effect of RRE on cell proliferation was measured by MTT assay and the effect of RRE on the production of nitric oxide (NO) was determined in the macrophage-like cell lines Raw264.7, C6 and peritoneal macrophages (pMQ). RRE inhibited the growth of tumor cells (e.g., B16, HOS). However, the effects of RRE on the production of NO varied with macrophage types. RRE had no effect on C6 cell growth and slightly increased the growth of Raw264.7 cells. In addition, treatment of normal pMQ with RRE enhanced NO production in a concentration-dependent manner, whereas RRE suppressed NO production at 50 μg/mL in both Raw264.7 and C6 cells. However, RRE suppressed NO production in LPS/IFN-γ-stimulated C6 cells. Overall, these results suggest that RRE elicits an antiproliferative property and differentially modulates NO production in various macrophages, and have a potential for therapeutic application.

Key words: Rhei Rhizoma, antiproliferative effect, nitric oxide, macrophage

INTRODUCTION

Recently, considerable attention has been focused on identifying naturally occurring chemopreventive substances capable of inhibiting, retarding or reversing the multi-stage carcinogenesis. One promising new source of therapeutic agents has been discovered in plant secondary metabolites, irregularly occurring compounds that characterize certain plants or plant groups. Immunotherapy with biological response modifiers (BRMs) has promise for the treatment of tumor and infectious disease (1). Immunomodulators which can stimulate the host's immune response have been useful as adjuvants and for nonspecific stimulation in animal tumor models (2). Among immune cells, macrophages have been known to play an important role in the nonspecific protection against a wide range of tumors and microorganisms (3,4). Macrophages also present antigen to lymphocytes during the development of specific immunity and serve as supportive accessory cells to lymphocytes. When activated, macrophages increase the phagocytic activity and release various materials such as cytokines and reactive intermediates and then carry out nonspecific immune responses (5,6). Especially, there has been great interest in the reactive nitrogen intermediate, nitric oxide (NO) which is considered to be a central molecule in the regulation of the immune response to tumors because of its cytotoxic effects (7,8).

Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new non-toxic therapeutic agents with anti-cancer properties. The biological and pharmacological properties of Korean traditional and Chinese herbs have begun to receive more attention in the scientific community as BRMs and have become a very important research focal point. Rhei Rhizoma extracts (RRE) is widely used in Southeast Asian a folk medicine to alleviate liver and kidney damage and RRE have been reported to attenuate metabolic disorders such as diabetic nephropathy, hypercholestrolemia and platelet aggregation (9,10). Recently, it has been shown that RRE have beneficial BRM such as anti-allergic or anti-hepatofibrosis effects (11,12). However, antiproliferative effects of RRE on cancer cells are still unknown. Moreover, there is no report on the effect of RRE on the function of tumor cells and primary normal cells, which possess similar characteristics. The purpose of this study was to assess the antiproliferative properties against various cancer cell lines and the effect of RRE on the production of NO in the macrophage families such as normal peritoneal macrophage (pMQ), mac-

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rophage-like cell lines including Raw264.7 and C6 cell lines in vitro.

MATERIALS AND METHODS

Preparation of RRE

The dried material (1 kg) was ground into a powder and subsequently extracted with MeOH (1:1) for 2 hrs at room temperature. The MeOH extract was evaporated under vacuum and suspended with D.W. (800 mL). The suspension was extracted with n-butanol and RRE was fractionated with D.W. Yields of water extracts were 16.5% (w/w) relative to the initial Rhei Rhizoma.

Cell culture and isolation of peritoneal macrophages

B16, HOS, Raw264.7 and C6 cells were obtained from ATCC (Manassas, VA) and were available in our laboratory. Cells were grown in RPMI 1640 (Gibco BRL Life Technologies, Inc., Rockville, MD) containing 10% (v/v) fetal bovine serum (FBS) (Gibco BRL) at 37°C, 5% CO_2. Thioglycollate-elicited peritoneal exudate cells were obtained from C57BL/6 mice (8 weeks old, Charles River Breeding Laboratories Atsugi, Japan) after an intraperitoneal injection of 1 mL Brewer Thioglycollate broth (4.05 g/100 mL, Difco Laboratories, Detroit, MI, USA) and a lavage of the peritoneal cavity with 5 mL of the medium 3～4 days later. The cells were washed twice and resuspended in RPMI-1640 containing 10% heat-inactivated FBS, penicillin (100 IU/mL) and streptomycin (100 μg/mL). The macrophages were isolated from the peritoneal exudate cells. The peritoneal exudate cells were seeded at densities of $5 \sim 6 \times 10^5$ cells/cm^2 on Teflon-coated petri dishes (100×15 mm), and the macrophages were allowed to adhere for 2～3 hr in a 5% CO_2 humidified atmosphere. The Teflon-coated petri dishes were prepared by spraying them with aerosolized Teflon (Fisher Scientific, Pittsburgh, PA, USA) followed by sterilization with UV light for 3 hr. The non-adherent cells were removed by washing the dishes twice with 10 mL of the pre-warmed medium, and dishes were incubated for 10 min at 4°C. The supernatants were then carefully removed and discarded, and the plates were washed once with pre-warmed Dulbecco's phosphate buffered saline (PBS) (Invtirogen Co., USA). Cold PBS (15 mL) containing 1.5% FBS was added followed by 0.3 mL of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages removed by rinsing them 10 times using a 10 mL syringe. The viability of the detached cells was assessed by trypan blue exclusion, and proportion of macrophages was determined after cytoplasmic staining them with acridine orange and observing them using fluorescence microscopy. The cell preparations were found to be more than 95% viable and contained more than 95% macrophages.

MTT assay for cell viability

Cell numbers were determined by hemocytometer counting and cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay adapted from Mosmann (13). Briefly, the cells were seeded in 96-well plates at suitable densities of cells per well with various concentrations of RRE. After an incubation period of 24 hrs, the enzyme activities of viable cells were measured by addition of MTT to each well. After 4 hrs of additional incubation, the amount of formazan was determined by absorbance at 540 nm using a microplate reader.

Nitrite determination

The cells were treated with the control media for 24 hrs in the presence or absence of various concentrations of RRE and the accumulation of nitrite in culture supernatant was measured using the assay system described by Ding et al. (14). 100 μL aliquots of culture supernatants were mixed with an equal volume of Griess reagent (mixture at 1:1 of naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4) and incubated at room temperature for 10 min. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

Statistical analysis

The data is expressed as a mean±SEM. The statistical difference between the groups was determined using a one-way analysis of variance (ANOVA) with Dunnett's *t*-test. A p value<0.05 was considered significant.

RESULTS AND DISCUSSION

Effect of RRE on the proliferation of tumor cells

The effect of RRE on the growth of different tumor cell lines was examined by measuring cell numbers using the MTT assay after treatment of the cultures with each extract for 24 hrs. Among these cell lines, HOS was the most sensitive to RRE treatment. The treatment of two cell lines (B16, HOS) with the extract resulted in decreased of the cell numbers (Fig. 1A and 1B). At high concentration (50 μM), RRE rapidly reduced the cell number of B16 cells and cell viability was significantly decreased by RRE treatment in a concentration-dependent manner. In contrast to these results, the extract did not have an inhibitory effect on both C6 and Raw264.7 cell proliferation (Fig. 2A and 2B). Treatment of Raw 264.7 with RRE resulted in the increase of cell viability at 50 and 100 μM. These data suggest that RRE has

Fig. 1. Effects of RRE on the viability of the B16 melanoma (A) and HOS osteoblastoma cells (B). (A) B16 (5×10^4 cells/well) was treated with RRE of various concentration for 24 hrs. (B) HOS (5×10^4 cells/well) were treated with RRE of various concentration for 24 and 48 hrs, respectively. The proliferation of cells was determined by MTT assay. Cell density was measured at 540 nm. The data represents the mean ±SE of quadruplicate experiments. $^{*}p<0.05$, $^{**}p<0.01$; significantly different from the control (no treatment).

Fig. 2. Effect of RRE on the viability of macrophages including Raw264.7 leukemia (A) and C6 glioma cells (B). Raw 264.7 (1×10^4 cells/well) and C6 (5×10^4 cells/well) were treated with various concentrations of RRE for 24 hrs. The proliferation of cells was determined by MTT assay. Cell density was measured at 540 nm. The data represents the mean± SE of quadruplicate experiments. $^{*}p<0.05$; significantly different from the control (no treatment).

a differential effect on cell types. In addition, the present data showing that RRE has an antiproliferative effect on non-immune cells such as B16 melanoma and HOS osteoblastoma are in keeping with the previous notion that RRE inhibited the proliferation of the renal carcinoma cell lines A498 (15). Our data also indicate that in macrophage families, RRE has different effects on cell cytotoxicity. When Raw264.7 was treated with RRE, cell proliferation increased and C6 had no effects. Although we used the tumor cell lines, the data in these studies that RRE might have a potential immunostimulatory effect as a candidate of BRM. Therefore, we examined the effect of RRE on the production of NO in macrophage-like cells lines and peritoneal macrophages.

Effect of RRE on nitric oxide production in macrophages

Since macrophages activation plays an important role in the host defense mechanism and NO is related to cytotoxic function of macrophages against a variety of tumors and microorganisms (16-18), we examined the effects of RRE on NO production using normal pMQs and Raw264.7 leukemia macrophages. As shown in Fig. 3, the treatment of the cells with RRE induced an increase in the production of NO by normal pMQs in dose-dependant manners, whereas NO production was decreased in Raw264.7 at 50 μM. We also observed similar results in C6 glioma cells (Fig 3C). To the best our knowledge, a dose response of RRE on NO production in various mononuclear cells is observed in macrophages. This differential effect of RRE may be explained by regulatory over-corrections by biosynthetic control mechanisms to low levels of growth inhibiting challenge (19). Based on these finding the present data suggest that RRE may have different effects on the function of normal and tumor macrophages.

Microglia are resident monocyte-lineaged cells in the brain. These cells defend the central nervous system against invading microorganisms and clear the debris from damaged cells. Recent biochemical and neuro-

A)

C)

B)

D)

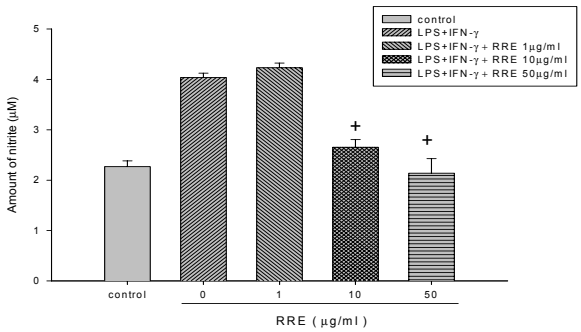

Fig. 3. Effect of RRE on the production of nitrite in Raw264.7 leukemia macrophages (A), normal pMQs (B) and C6 cells (C, D). Raw264.7 (1×10^4 cells/well) and pMQs (1×10^5 cells/well) were treated with various concentration of RRE for 24 hrs. C6 (5×10^4cells/well) were treated with various concentration of RRE for 24 hrs in the absence (C) or presence (D) of LPS plus IFN-γ. Culture supernatants were collected and the levels of nitrite were measured as described in materials and methods. The data represents the mean$\pm$SE of quadruplicate experiments. $^{*}p<0.05$, $^{**}p<0.01$; significantly different from the control (no treatment). $^{+}p<0.05$; significantly different from the treatment of LPS plus IFN-γ.

biological studies have further indicated that they significantly affect the pathological state and/or regulate the regenerative state and remodeling of the brain by producing a variety of biologically active molecules including radicals such as superoxide anion and NO (20). The immunomodulating effects of RRE on NO production also are examined in microglia, C6 glioma cells. As shown in Fig. 3C, RRE has no effects on the production of NO in microglia, whereas RRE suppressed NO production in LPS/IFN-γ-stimulated microglia (Fig. 3D). Under pathological conditions, excessive formation of NO is associated with tissue damage observed in arthritis, type 1 diabetes, septic shock and Alzheimer's disease (20). Therefore, inhibitory effects of RRE on NO production induced by LPS plus IFN-γ in C6 cells could provide the opportunity to develop a new therapeutic agent for neuronal disease.

At the present time the mechanisms by which RRE has a biphasic response to microglia are not clear. Further studies are needed regarding the identification of active compounds and their mechanism of action. However, the present data suggest that RRE has biological effects on various cell types in direction to positive effects for human health and might be useful for possible therapeutic applications.

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