In vitro treatment of chicken peripheral blood lymphocytes, macrophages, and tumor cells with extracts of Korean medicinal plants

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Abstract

A variety of different medicinal plants have traditionally been used in Asian cultures as medicinal plants to enhance immunity and treat cancers. However, limited information exists on the underlying mechanisms responsible for these immune enhancing properties. The current investigation was conducted to examine the effects of methanol extracts of 3 Korean indigenous plants (dandelion root, mustard leaf, and safflower leaf) on various in vitro parameters of innate immunity (peripheral blood lymphocyte proliferation, nitric oxide production by macrophages, and free radical scavenging activity) and tumor cell growth. All plant extracts inhibited tumor cell growth and exerted antioxidant effects compared with vehicle controls. In addition, safflower leaf extract stimulated lymphocyte proliferation and mustard leaf induced nitric oxide production. These results demonstrate, for the first time, that traditional Korean medicinal plant extracts are effective in enhancing innate immunity and suppressing tumor cell growth.

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1. Introduction

Widespread application of prophylactic antibiotics has been successful in reducing infection-related morbidity and mortality in humans and animals. However, the increasing dependence of feed-added chemicals for animal production and the public anxiety over the emergence of antibiotic-resistant microbial pathogens are driving a new emphasis on the development of alternative methods of disease control.

In this regard, recent progress has been achieved on the identification and commercial application of plant phytonutrients possessing immunoenhancing activities, particularly in boosting innate immunity against infectious diseases and tumors [1-5]. For example, dandelion leaf and shiitake mushroom have been shown to stimulate lymphocyte proliferation and inhibit tumor cell growth [6-8], and a crude extract of \textit{Withania somnifera} (an Ayurvedic medicinal herb commonly known as ashwagandha) exhibited immunoregulatory effects in mice [9]. Some plant extracts kill tumor cells by blocking metabolic pathways or inducing apoptosis in vitro [10,11]; others have been shown to inactivate free radicals [12]. Oxidative stress, resulting from an imbalance between pro- and antioxidant systems in favor of the former, largely contributes to immune system deregulation and complications [13].
A diverse array of medicinal plants have traditionally been used in Korea to enhance innate immunity and treat cancers, and preliminary studies have documented the metabolic effects of some of these plants. Dietary supplementation with dandelion extract was reported to improve lipid metabolism and prevent diabetic complications due to lipid peroxidation and free radicals in diabetic rats [14]. Safflower has been known to improve the repair of fractured bone and lower blood lipid levels [15, 16]. However, few studies have characterized the effects of these medicinal plants on immunity [17], particularly in the chicken, an economically important food animal in which innate immune responses have been relatively well defined [18-21]. The current investigation was conducted to examine the potential immunoenhancing properties of extracts derived from dandelion root, mustard leaf, and safflower leaf on various parameters of chicken innate immunity and tumor cell growth.

2. Methods and materials

2.1. Preparation of methanol extracts

Methanol extracts of dandelion root (Taraxacum oficinale), mustard leaf (Brassica juncea var integrifolia), and safflower leaf (Carthamus tinctorius) were obtained from the National Rural Resources Development Institute (Suwon, South Korea). Extraction was carried out by adding 100 mL of 80% methanol to 30 g of the sample and vigorous shaking for 48 hours at room temperature. The process was repeated 3 times, the pooled extracts were rotary evaporated (Eyela, Irvine, Calif), and the residue was freeze-dried and stored at -80°C. Before use, the samples were dissolved at 125 μg/mL in phosphate-buffered saline (PBS) for cell culture assays or in methanol for antioxidant activity assay and sterilized by membrane filtration through a 0.2-μm filter (Nalgene, Rochester, NY).

2.2. Lymphocyte proliferation

Specific pathogen-free White Leghorn SC inbred chickens (Hy-vac, Adel, Iowa) were maintained at the Beltsville Agricultural Research Center and fed ad libitum with a standard diet formulated to meet the nutrient requirements as recommended by the National Research Council [22]. At 3 weeks of age, animals were killed by cervical dislocation and bled by cardiac puncture using a heparinized syringe. All experiments were approved by the Animal and Natural Resources Institute Institutional Animal Care and Use Committee (IACUC). Peripheral blood lymphocytes (PBL) were prepared as described [23], and lymphocyte proliferation was carried out as previously reported [3, 24]. In brief, isolated PBL were adjusted to 1 x 10^6 cells/mL in enriched RPMI-1640 medium without phenol red (Sigma, St. Louis, Mo) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). The PBL suspension (100 μL/well) was cultured with 100 μL of plant extracts, 5.0 μg/mL of concanavalin A (Con A, Sigma) as a positive control, or PBS as a negative control in 96-well flat-bottom plates at 41°C in a humidified incubator (Forma, Marietta, Ohio) with 5% carbon dioxide/95% air for 48 hours. Cell proliferation was determined with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8, Cell-Counting Kit-8, Dojindo Molecular Technologies, Gaithersburg, Md) as described [25]. Cell numbers were measured by optical density (OD) at 450 nm using a microplate reader (BioRad, Richmond, Calif).

2.3. Inhibition of tumor cell growth

RP9 tumor cells, which are commonly used as a target cell for chicken NK cell activity [26], were cultured at 1 x 10^6 cells/mL (100 μL/well) with plant extracts (100 μL/well), 1.0 μg/mL of recombinant human tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, Minn) as a positive control, or PBS as a negative control in 96-well microtiter plates at 41°C in 5% carbon dioxide for 48 hours. After incubation, the cells were radiolabeled for 4 hours with 0.25 μCi/well of [3H]-thymidine (Perkin Elmer, Boston, Mass), harvested using a semiautomated cell harvester (Tomtec, Orange, Conn), and cellular radioactivity was determined using a liquid scintillation counter (1450 Microbeta Wallac Trilux, Perkin Elmer).

2.4. Nitric oxide production by macrophages

HD11 macrophages [27] were cultured at 1 x 10^7 cells/mL (100 μL/well) with plant extracts (100 μL/well), 1.0 μg/mL of recombinant chicken interferon-γ (IFN-γ) as a positive control [28], or PBS as a negative control in 96-well microtiter plates at 41°C in 5% carbon dioxide for 24 hours. After incubation, 100 μL of cell culture supernatants were transferred to clean 96-well plates, mixed with 100 μL of Griess reagent (Sigma), and the plates were incubated for 15 minutes at room temperature. Optical density at 540 nm was measured, and the nitrite concentration was determined using a standard curve generated with known concentrations of sodium nitrite.

2.5. Scavenging activity of a,a-diphenyl-β-picrylhydrazyl (DPPH) free radical

Two milliliters of plant extracts, 100 μg/mL of vitamin C as a positive control, methanol vehicle control were mixed with 1.0 mL of 0.35 mmol/L a,a-diphenyl-β-picrylhydrazyl (DPPH) in methanol. The mixture was shaken vigorously at room temperature for 30 minutes, and OD was measured at 517 nm against the sample blank control with DPPH alone [29]. The DPPH scavenging activity was calculated according to the following formula: [(OD517control – OD517sample) / OD517control] x 100%.

2.6. Statistical analysis

All samples were analyzed in triplicate, and data were expressed as means ± SEM using InStat software.
Analysis of variance was used to test for differences between groups. The Tukey-Kramer multiple range test was used to determine the significance of differences between the mean values of the treatment groups at the level of $P < 0.05$ [30].

3. Results

3.1. Lymphocyte proliferation

Safflower leaf extract significantly enhanced PBL proliferation compared with the PBS negative control (Fig. 1) ($P < 0.05$). The increase in lymphocyte proliferation induced by the safflower leaf extract was approximately 50% of that produced by the ConA positive control. In contrast, methanolic extracts of dandelion root and mustard leaf did not stimulate PBL proliferation.

3.2. Tumor cell growth

Extracts of dandelion root, mustard leaf, and safflower leaf significantly reduced the growth of RP9 tumor cells compared with the PBS negative control (Fig. 2) ($P < 0.05$). The growth inhibitory effect of all 3 extracts was quantitatively identical to that of the TNF-$

3.3. Nitric oxide production

Mustard leaf extract significantly induced nitric oxide (NO) production by HD11 macrophages compared with the PBS negative control (Fig. 3) ($P < 0.05$). The increase in NO stimulated by the mustard leaf extract was approximately 50% of that produced by the ConA positive control. In contrast, methanolic extracts of dandelion root and mustard leaf did not stimulate PBL proliferation.
55% of that produced by the IFN-γ positive control. In contrast, methanol extracts of dandelion root and safflower leaf did not stimulate NO production.

4. Discussion

Cell culture systems have provided a wealth of information on the biological effects of phytochemicals from fruits and vegetables and on the mechanisms through which diets high in fruits and vegetables may reduce the risk of chronic diseases [32,33]. Previous studies have demonstrated the stimulatory effects of natural foods and herbal products on lymphocyte proliferation [34,35]. However, little is known about the biological effects of the particular medicinal plants examined in this study, and no reports have described their effects on innate immunity in terms of the 3 parameters examined herein (PBL proliferation, NO production, free radical scavenging activity). Our results demonstrated that all of these parameters were affected by at least one of the extracts, and free radical scavenging activity was increased by all 3 extracts. The stimulatory effect of safflower leaf extract on PBL proliferation corroborates a prior study examining the effect of safflower seed oil on monocytes [36].

Host immune function is critically important in the response to tumorigenesis [6]. Our results demonstrated that the antitumor effects of the 3 extracts were similar to that of TNF-α, one of the most potent antitumor molecules known [37]. In a previous study, dandelion leaf was found to inhibit tumor cell growth [7], and safflower petals also showed potential as an anticancer agent [38]. The antitumor effects of safflower have been speculated to be due to its ability to stimulate lymphocyte proliferation of cancer-specific cytotoxic T cells [36]. Taken together with our current observations, these results suggest that the pharmacologic agent(s) present in dandelion and safflower are distributed throughout various parts of the plants.

Macrophages play a significant role in host defense against infectious agents and tumors mediated, in part, by elaboration of soluble inflammatory molecules, such as NO [6]. In this study, the extract of mustard leaf significantly increased NO production by HD11 macrophages compared with the vehicle control. Interestingly, safflower leaf extract did not stimulate NO production, although safflower petals have been shown to activate macrophages [38]. The involvement of NO during innate immune response, macrophage-mediated killing, or direct growth inhibition of microorganisms and tumor cells has been previously documented [6,21,31,39].

In conclusion, we demonstrate that methanol extracts of 3 particular traditional medicinal plants commonly used in Korea are capable of affecting various in vitro parameters of chicken innate immunity and inhibition of tumor cell growth. The results obtained with the individual cell types in culture provide important clues as to their effects on the immune system. Further studies on the nature of the immunomodulating activities of these and other phytoneutrients will facilitate the development of novel strategy against infectious diseases and tumors in humans and commercial food animals.

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