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The Effect of *Salvia plebeia* on Murine Macrophage-mediated Cytotoxicity

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Abstract – Macrophages play an important role in host defense against tumors by killing tumor cells. Our work is directed toward studying the effect of the extracts of *Salvia plebeia* on induction of antitumor activity in macrophages, since it has been usezd as a folk-medicine for the treatment of hepatitis and tumors. The ability of macrophage treated with the plant extracts to inhibit the growth of tumor cells was assessed. The Extracts of the plant induced antitumor activity and could enhance the tumoricidal activity of macrophages when used in combination with IFN-γ. These results suggest that *Salvia plebeia* extract contain immunomodulatory factors responsible for the induction of the antitumor activity.

Key words – *Salvia plebeia*, antitumor activity, macrophage.

Introduction

Macrophages play an important role in defense against virus infection by killing virus infected cells (extrinsic resistance) and by limiting viral replication within themselves (intrinsic resistance)(Morahan *et al.*, 1984; Morahan *et al.*, 1985). The cytotoxic (cytostatic) activity of macrophages is also involved in resistance to tumors (Cohn *et al.*, 1978; Keller *et al.*, 1976). Although the mechanisms by which macrophages kill tumor cells has been studied in some detail, the activity of macrophages against cancers is less well understood.

Macrophages can be activated by a variety of agents and some of these have also been shown to increase resistance to tumors when administered *in vivo* (Campbell *et al.*, 1980). Treatment of mice with *Propionibacterium acnes* (formerly *Corynebacterium parvum*) has been shown to result in increased resistance to tumor cells. This increased resistance may be partially due to increased macrophage cytotoxic activity.

Salvia plebeia is an annual herb of Labiatae, which has been used in folk medicine for the treatment of hepatitis and tumors (Kan, 1971). In addition, one of the *Salvia* species showed antiviral activity (Tada *et al.*, 1994). Although some constituents have been isolated from *Salvia plebeia*, there are no reports on their biological activity. In this study we have examined whether the extracts of *Salvia plebeia* can enhance macrophage antitumoral activity and have investigated their immunomodulatory activity *in vitro*.

Experimental

Mouse and tumor cell – The mouse strains used in this study was CD-1, which was obtained from the Animal Center (Seoul National University). For *in vitro* use, KB tumor cell (epidermoid carcinoma) was grown in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml)(RPMI-

FBS).

Chemicals and antibodies – Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO). Recombinant murine interferon (IFN)-γ and polyclonal anti-mouse tumor necrosis factor (TNF) antibody (1×10^6 neutralizing units/ml) were purchased from Genzyme Corporation (Boston, MA).

Plant extraction – Plant materials were harvested in Kyunggi-do, Korea in July 1994. Dried *Salvia plebeia* aerial part (500 g) was extracted with 2 L of methanol at 50 °C for 5 hours. After filtration, the filtrate was evaporated to dryness and then the residue was extracted with 2 L of water by reflux at 100 °C for 10 hours. After refiltraion, the resulting extract was concentrated to 50 ml (fraction 1: Fr. 1). The methanol extract was partitioned between water (500 ml) and methylene-chloride (500 ml). This water layer was extracted with *n*-buthanol and the resulting water layer was used as the fraction 2 (Fr. 2).

Isolation of inflammatory peritoneal macrophages – Thioglycolate-elicited peritoneal exudate cells were obtained from 4-6 weeks old CD-1 male mice by intraperitoneal injection of 1 ml Brewer thioglycolate broth (4.05 g/100 ml)(Difco labs, Detroit, MI) and lavage of the peritoneal cavity with 5 ml of medium 3-4 days later. The cells were washed twice and resuspended in RPMI-FBS. Macrophages were isolated from the peritoneal exudate cells (Klimetzek *et al.*, 1980). Peritoneal exudate cells were seeded at densities of $5\text{-}6\times10^5$ cells /cm^2 on teflon-coated petri dishes (100×15 mm) and the macrophages were allowed to adhere for 2-3 hours in 5% CO_2 incubator. Teflon-coated petri dishes were prepared by spraying with aerosolized teflon (fisher Scientific, Pittsburgh, PA) and sterilizing with ultraviolet light for 3 hours. The nonadherent cells were removed by washing the dishes twice with 10 ml prewarmed RPMI-1640 and the dishes were incubated for 10 min at 4 °C. The supernatants were then carefully removed and discarded and the plates were washed once with prewarmed Dulbecco's Phosphate Buffered Saline (PBS) (Gibco). Cold PBS (15 ml) containing 1.5% FBS (PBS-FBS) was added. Then 0.3 ml of 0.1 M EDTA (pH 7.0) was added. The plates were incubated for 15 min at room temperature and the macrophages were removed by rinsing 10 times using a 10 ml syringe. The detached cells were washed once with PBS-FBS and resuspended in RPMI-FBS. The viability of the detached cells was assessed by trypan blue exclusion and the proportion of macrophages was determined after cytoplasmic staining with acridine orange and examination using a fluorescence microscope. Cell preparations were >95% viable and contained 90% macrophages.

Macrophage-mediated cytotoxicity – The assay for macrophage cytotoxicity is based on an assay described previously (Mosmann, 1983). Briefly, macrophages (1×10^5 cells/well) were plated into 96-well microtiter plates (Nunc, Denmark) and incubated with various doses of plant extracts. At the extracts dosages that we employed, no toxicity was observed. In some experiments, antibody to cytokine or inhibitor of metabolic pathways was included along with plant extracts. Then B16 tumor cells (1×10^4 cells/well) were added, and the plates were placed in a humidified 37 °C incubator for 48 hours. After 48 hours, 20 μl of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) was added to each well and incubated for 4 hours. At the end of the incubation, supernatants were discarded and 200 μl of dimethyl sulfoxide was added to dissolve the dark blue formazan crystals. Plates were shaken gently for 15 min on rotary shaker, and then read at 540 nm (A_{540}) on Molecular Device microplate reader (Menlo, CA). Data were expressed as the mean absorbance values (optical density: OD) of quintuple samples and computed as

the mean percent cytotoxicity where:

% Cytotoxicity = 100 - {[(OD macrophages + tumor target) - (OD macrophages)/OD tumor target] × 100}.

Result and Discussion

A number of compounds have been implicated in macrophage cytotoxicity including H_2O_2, TNF, and NO (Mavier *et al.*, 1984; Decker *et al.*, 1987; Hibbs *et al.*, 1987). To determine whether these compounds were involved in cytotoxicity mediated by extracts of *Salvia plebeia*, we attempted to abrogate cytotoxicity by inhibiting their production or by neutralizing their activity. In these experiments we employed extracts at a concentration of 20 µg/ml since preliminary experiments indicated that higher doses were cytotoxic to cells. The general design of these experiments was to pretreat cells with plant extracts (Fr. 1 and Fr. 2) in the presence of the inhibitors or antibodies, and to assess cytotoxic activity. At the concentrations employed none of the inhibitors or antibodies affected the growth of the tumor cells (data not shown). The role of H_2O_2 was evaluated by inactivating it with catalase, the role of TNF by neutralizing antibody, the role of superoxide anion and NO with superoxide dismutase or N^G-monomethyl-L-arginine, respectively.

As shown in Table 1 and 2, both Fr. 1 and Fr. 2 stimulated macrophage cytotoxicity to the same extent as LPS and IFN-γ. We have observed similiar results at various ratios of target cells/effector cells. In addition, none of the other fractions from *Salvia plebeia* (methanol extract, methylene chloride extracts or buthanol extract) was effective in inducing activities of macrophages (data not shown). Cytotoxic activities of macrophages were enriched into Fr. 1. However, the addition of the inhibitors or antibodies did not inhibit the cytotoxic activity of activated macrophages against tumor cells (Table 1 and 2).

Table 1. Inhibition of tumoricidal activity of Fr.1-activated macrophages by antibodies or inhibitors.

Treatment[a]	% Cytotoxicity of target cell (KB)
None	22.71±1.68
Fr.1 (20 µg/ml)	71.90±8.09
Fr.1+anti-TNF-α (500 U/ml)	70.00±2.53
Fr.1+NMMA[b] (0.5 mM)	69.95±3.05
Fr.1+SOD[c] (400 U/ml)	68.66±4.00
Fr.1+catalase (500 U/ml)	70.55±0.45
LPS (100 ng/ml)+IFN-γ (100 U/ml)	82.95±1.59

[a] Peritoneal macrophages were cocultured for 40 hours with target at an effector/target cell ratio of 10:1 in medium alone or in medium supplemented with Fr. 1 in the absence or presence of various antibodies or inhibitors. The results are mean±S.D. of quintuplicates from representative experiment.
[b] NMMA: N^G-monomethyl-L-arginine
[c] SOD: Superoxide dismutase.

Table 2. Inhibition of tumoricidal activity of Fr.2-activated macrophages or inhibitors.

Treatment[a]	% Cytotoxicity of target cell (B16)
None	22.71±1.68
Fr.1 (20 µg/ml)	50.30±3.66*
Fr.1+anti-TNF-α (500 U/ml)	52.00±1.22
Fr.1+NMMA[b] (0.5 mM)	51.00±3.05
Fr.1+SOD[c] (400 U/ml)	50.11±5.22
Fr.1+catalase (500 U/ml)	56.21±5.47
LPS (100 ng/ml)+IFN-γ (100 U/ml)	82.95±1.59

[a] Peritoneal macrophages were cocultured for 40 hours with target at an effector/target cell ratio of 10:1 in medium alone or in medium supplemented with Fr. 2 in the absence or presence of various antibodies or inhibitors. The results are mean±S.D. of quintuplicates from representative experiment.
*Significantly different from control (no treatment); $P<0.001$.

Thus, reactive oxygen species, NO, and TNF did not appear to be involved in the antitumor activity of plant extracts-stimulated macrophages. In addition, we have observed that both fractions did not protect macrophages from infection with herpes simplex virus type-1 (unpublished data). Since a number of different activators have been identified and multiple cytotoxic effector molecules have been implicated in ma-

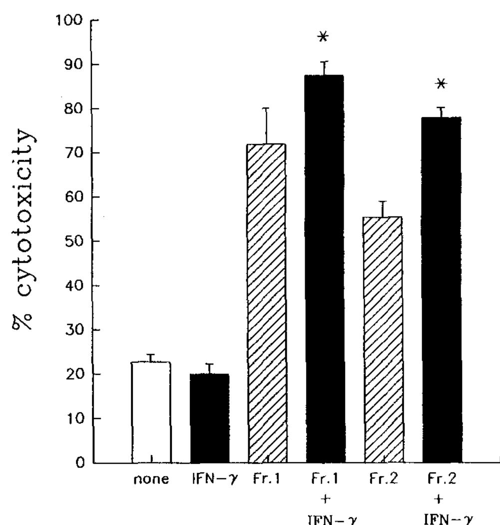

Fig. 1. Peritoneal macrophages were cocultured for 40 hours with target at an effector/taregt cell ratio of 10:1 in medium alone or in medium supplemented with each fraction in the absence or presence of IFN-γ. Data Presented are the mean±S.D. of quintuplicates from a representative of two experiments.
*Significantly different from IFN-γ alone, $p<0.01$ to $p<0.005$.

crophage-mediated cytotoxicity (Adams, 1980; Onozaki *et al.*, 1985), these results suggested that fraction treatment may lead to different modes of macrophage activation and consequently to alternative pathways of macrophage-mediated cytolytic activity.

Since murine macrophages may be activated for tumor cytotoxicity by IFN-γ in synergy with LPS, Lipid A or a variety of other microbial and nonspecific agents (Herriott, *et al.*, 1987), we next determined whether IFN-γ synergized with fractions induced the activation process for tumor cytotoxicity in macrophages. Results (Fig. 1) revealed that whereas IFN-γ alone failed to induce macrophages activation, macrophages stimulated with IFN-γ and fractions were cytotoxic to KB tumor cells. These results strongly suggested that fractions might be involved which synergized with IFN-γ to render macrophages cytotoxic. Recent reports demonstrated that IL-2 synergized with IFN-γ to mediate activation of murine macrophages and macrophage-like cell lines for tumor cytotoxicity (Cox *et al.*, 1990, 1991). In addition, both IL-3 and GM-CSF synergized with LPS for human monocyte-mediated cytotoxicity of a TNF-sensitive target (Cannistra, *et al.*, 1988).

From our current findings, it seems that plant extracts contain immunoregulatory substances having a bioactivity similar to IL-2, IL-3, or GM-CSF. Presently, we are in the process of purification and characterization of plant extracts to determine whether characterized agents have a similar biological activity.

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