Immuno­modulatory action of citrus auraptene on macrophage functions and cytokine production of lymphocytes in female BALB/c mice

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The modifying effects of auraptene isolated from the peel of citrus fruit (Citrus natsudaidai Hayata) on macrophage and lymphocyte functions were investigated in mice. Female BALB/c mice were gavaged with auraptene at a dose of 100, 200 or 400 mg/kg once a day for 10 consecutive days. Glucose consumption of peritoneal macrophages was significantly higher than that in the control group (P < 0.05–0.001) in auraptene-treated mice at all doses at 24, 48 and 72 h incubation except for mice given 200 mg/kg auraptene at 24 h incubation. Activity of acid phosphatase in peritoneal macrophages was significantly increased in mice treated with auraptene at a dose level of 100 mg/kg (P < 0.001). Activity of β-glucuronidase in peritoneal macrophages in the auraptene-treated mice at all doses was significantly higher than that in the control group (P < 0.001), but there was no significant difference in lactate dehydrogenase activity of peritoneal macrophages at any dose. Interleukin (IL)-1β production of peritoneal macrophages in the auraptene-treated mice at all doses was significantly higher than that in the control group (P < 0.005–0.0001). Tumor necrosis factor α production of peritoneal macrophages in mice gavaged with auraptene at a dose of 200 mg/kg was significantly higher than that in the control group (P < 0.05). Auraptene did not affect proliferation of spontaneous splenic lymphocytes in mice at any dose. Stimulation indices in mice given auraptene at a dose of 200 mg/kg were significantly higher than that in the control group (P < 0.05). When splenetic lymphocytes were cultured without concanavalin A (Con A), IL-2 and interferon (IFN) γ productions were not detectable in the supernatant. However, IL-2 and IFN production stimulated by Con A were significantly increased in mice gavaged with auraptene at dose levels of 100 and 200 mg/kg (P 0.05–0.001). Auraptene did not enhance spontaneous IL-4 production by splenocytes. There was no significant difference in IL-4 production of splenic lymphocytes stimulated by Con A in all groups. These findings might suggest that oral administration of citrus auraptene effectively enhanced macrophage and lymphocyte functions in mice.

Introduction

Epidemiological data suggest that ingestion of some constituents from vegetables and fruits may contribute to a reduction in cancer incidence in humans (1–3). In experimental studies, several investigators reported that ingestion of extracts and several components from fruits and vegetables suppress carcinogenesis (4–9). It is well known that dietary factors play an important role in enhancement of health status and physical strength in humans. On an earlier occasion, foods were evaluated by both nutritional function (primary function) and gustatory function (secondary function). Recently, it has been shown that certain foods have a host defense function related to the immune system (10–13) and anti-oxidation (14,15) and anti-tumor (16,17) activity. The immune system plays an important role in physical and chemical carcinogenesis (18,19) and in tumor-bearing hosts (20). The role of host immune function has become increasingly important in our understanding of the mechanisms that are involved in the body’s ability to prevent cancer. Although the inter-relationship between diet, immune function and carcinogenesis is not clear, there is increasing evidence that dietary alteration of the host’s immune functions is a key component of chemoprevention (21–23).

Macrophages, lymphocytes (T and B cells), dendritic and Langerhans’ cells and natural killer (NK) cells are important cells for the immune system. Macrophages play a major role in inflammation, repair, humoral and cellular immunity and metabolic and neoplastic disease processes. Cytokines, being messenger molecules of the immune system, modulate natural immunity. These include interleukin (IL)-1β, IL-2, IL-4, tumor necrosis factor (TNF) α and interferon (INF) γ. INFs protect against viral infections, whereas IL-1 and TNFα initiate non-specific inflammatory responses. Certain cytokines [IL-2, IL-4, IL-5, IL-12 and tumor growth factor (TGF)-β] regulate lymphocyte growth, activation and differentiation. Among them, IL-2 and IL-4 favor lymphocyte growth and differentiation. INFγ, TNFα and β, IL-8 and migration inhibitory factor activate inflammatory cells and serve to activate the functions of non-specific effector cells. IL-1, one of the pro-inflammatory cytokines, activates T and B cells. IL-1β is produced by monocytes and/or macrophages, etc. IL-2, produced by activated T cells, macrophages, NK cells, etc., are able to induce proliferation of T and B cells and antibody production by B cells. IL-4, known to activate monocytes and/or macrophages, is produced by T helper (Th)2 cells or mast cells. IL-4 induces the anti-tumor function of macrophages and reduces the production of inflammatory cytokines. TNFα, produced by activated macrophages and lymphocytes, enhances the cytotoxic action of macrophages. INFγ, produced by

Abbreviations: APH, acid phosphatase; Con A, concanavalin A; GLU, β-glucuronidase; INF, interferon; IL, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NK, natural killer; PEC, peritoneal exudate cells; SI, stimulation index; TGF, tumor growth factor; Th, T helper; TNF, tumor necrosis factor.
stimulated T cells, inhibits cell proliferation, has an anti-tumor effect, activates macrophages and induces cell differentiation. Thus, cytokines have multiple biological functions. It is known that several cancer chemopreventive agents can modulate immune function (11,13,24–26). Non-specific immune stimulation has a protective effect against chemically induced colon carcinogenesis (27). Recently, it was reported that the cytokine TGF-β1, known to play a key role in the control of cell growth, inhibits experimental colon carcinogenesis (28). Several Indian Ayurvedic products can reduce chemically induced mammary tumors in rats without any toxicity (29). Such Indian Ayurvedic agents are also able to induce differentiation of several tumor cell lines (30,31). Our previous experiments (12,32–34) demonstrated that these agents enhance macrophage function and lymphocyte responsiveness in mice. A known cancer chemopreventive agent, dialyl trisulfide, can augment activation of T cells and enhance the anti-tumor function of macrophages (35). Also, it is reported that curcumin may exert its anti-carcinogenic activity through enhancement of immune function, such as the antibody response (36). Thus, certain cancer chemopreventive compounds may exert their inhibitory properties against cancer development through modulation of immune function.

An antioxidant auraptene isolated from the peel of citrus fruit (Citrus natsudaidai Hayata) has been reported to have chemopreventive effects on chemically induced carcinogenesis (4,9,15). Dietary administration of auraptene significantly increased the activities of detoxification (phase II) enzymes, such as quinone reductase and glutathione S-transferase, in the liver and colon of rats. In addition, expression of cell proliferation biomarkers, such as ornithine decarboxylase activity and polynucleo biosynthesis, in the colonic mucosal epithelium was significantly inhibited by dietary feeding of auraptene. These biological functions of auraptene may contribute to its anti-tumorigenic effect. However, a modulatory effect of auraptene on immune function has not been investigated.

In the present study, we have investigated whether gastric intubation of citrus auraptene modifies (enhances) immune function, especially macrophage function, splenic lymphocyte proliferation and cytokine production, in mice. Macrophage function was evaluated by measuring glucose consumption, lysosomal enzyme activity and production of IL-1β and TNFα by peritoneal macrophages as an indicator. Mitogenic response and production of IL-2, IFNγ and IL-4 of spleen cells were examined for assessment of lymphocyte function.

Materials and methods

Animals and isolation of auraptene
A total of 76 female BALB/c inbred mice, 7 weeks old (weighing 16–21 g), obtained from Japan SLIC (Hamamatsu City, Japan), were used for this experiment. They were housed, four to five per cage, with a pelleted basal diet (CE-2; Clea Japan, Tokyo, Japan) and water ad libitum, in an animal room under a 12 h light/dark cycle at a temperature of 22 ± 1°C and a humidity of 60 ± 5%. After 1 week acclimatization, they were used for the experiment.

To isolate auraptene, natsumikan (Wakayama Prefecture) were harvested in 1998 and whole parts (4.8 kg fresh weight) were processed using a FMC Citrus Juice Extractor (FMC Co., Rockland, ME) to give the cold-pressed juice. The whole parts of the juice were then left to stand to remove tissue fragments. The cell suspensions were centrifuged (600 g for 10 min), resuspended in 10% fetal calf serum, RPMI 1640 medium, and incubated at 37°C with 5% CO2 in humidified air for 24 h with 50 µl of lipopolysaccharide (LPS) (10 µg/ml, E.coli 050: B5; Sigma Chemical Co.). The results are expressed as percent glucose consumption, calculated from the following formula: [1 – (glucose content in culture medium with macrophages/glucose content in culture medium without macrophages)] × 100.

Assay of enzyme activities
Enzyme activities in peritoneal macrophages were determined by the method reported previously (12). The intracellular activities of acid phosphatase (AP), β-glucuronidase (GLU) and lactate dehydrogenase (LDH) were measured by the p-nitrophenyl phosphate method (Wako Co.), with the GLU kit (Sigma Chemical Co., St Louis, MO) and by the tetrazolium salt method (Wako Co.), respectively. The APH activity is expressed as IU per 2×10⁶ cells, GLU activity is expressed as IU per 4×10⁵ cells and LDH activity is expressed as IU per 1×10⁵ cells.

Production of IL-1β and TNFα
Macrophages (100 µl, 1×10⁵ cells) were cultured at 37°C with 5% CO2 in humidified air for 24 h with 50 µl of lipopolysaccharide (LPS) (10 µg/ml, E.coli 050: B5; Sigma Chemical Co.). IL-1β and TNFα activities in culture supernatants were measured by ELISA using commercial kits for mouse IL-1β and TNFα (Endogen, Woburn, MA). The samples were frozen and stored at −80°C until use.

Mitogenicity assay
The proliferation of spleen cells was determined by the method reported previously (32). The experiments were done in triplicate. Stimulation index (SI) was calculated using the equation: SI = mean optical density of cells stimulated with concanavalin A (Con A) + mean optical density of cells not stimulated with Con A.

Production of IL-2, IFNγ and IL-4
Spleen cell suspension (100 µl) at a concentration of 4×10⁵ cells/ml was incubated at 37°C with 5% CO2 in humidified air for 48 h with Con A (5 µg/ml). IL-2, IFNγ and IL-4 activities in culture supernatants were determined using an ELISA kit (Endogen). The samples were frozen and stored at −80°C until use.

Treatment with auraptene
Auraptene was suspended in 5% gum arabic (Wako Co., Osaka, Japan) and gavaged to mice at a dose of 100, 200 or 400 mg/kg/day for 10 consecutive days. Control mice were given 5% gum arabic as the vehicle (0.1 ml/10 g body wt). The animals were killed by bleeding 48 h after the last administration under ether anesthesia for the following experiments. Forty mice were used for determination of peritoneal macrophages and 36 for preparation of splenic lymphocytes.

The experimental protocol, animal care and treatment were approved by the Committee for Animal Studies at Gifu Pharmaceutical University and that at Kanazawa Medical University.

Isolation of macrophages
All procedures were conducted under aseptic conditions. Each group consisted of 10 mice. Mice were killed by bleeding under ether anesthesia and peritoneal exudate cells (PEC) were obtained by i.p. injection of Hanks’ solution (Nissui Seiyaku Co., Tokyo, Japan). The PEC were pooled in each group. Macrophages in the PEC suspension were isolated by the cell adhesion method. The PEC were suspended in RPMI 1640 medium (Nissui Seiyaku Co.) containing 10% heat-inactivated fetal calf serum (Gibco Laboratories Life Technologies, New York, NY) and incubated in a culture plate (Corning Laboratory Sciences Co., New York, NY) for 2 h at 37°C in 5% CO2 in an incubator. After removing non-adherent cells by washing the plate with Hanks’ solution, the adherent cells were harvested from the bottom using a rubber policeman and resuspended in 10% fetal calf serum, RPMI 1640 medium. The cells were used for experiments as resident peritoneal macrophages at a concentration of 2×10⁶ cells/ml. Cell viabilities checked by the trypan blue dye exclusion test were >95%.

Preparation of splenic lymphocytes
All procedures were conducted under aseptic conditions. Each group included nine mice. Mice were killed by cervical dislocation under ether anesthesia and a single cell suspension was prepared by pressing the spleen between two slide glasses. The cell suspensions were passed through a 200 gauge stainless steel sieve and then left to stand to remove tissue fragments. The cell suspensions were centrifuged (600 g for 10 min), resuspended gently in fetal calf serum/RPMI 1640. The cell suspensions were adjusted to 4×10⁶ viable cells/ml. The viability of splenocytes determined by trypan blue dye exclusion was >95%.

Assay of glucose consumption in peritoneal macrophages
Glucose consumption in peritoneal macrophages was measured as described elsewhere (32). Glucose remaining in the peritoneal macrophage culture supernatant was determined using a commercial kit (Glucose B-Test; Wako Co.). The results are expressed as percent glucose consumption, calculated from the following formula: [1 – (glucose content in culture medium with macrophages/glucose content in culture medium without macrophages)] × 100.

Assay of enzyme activities
Enzyme activities in peritoneal macrophages were determined by the method reported previously (12). The intracellular activities of acid phosphatase (APH), β-glucuronidase (GLU) and lactate dehydrogenase (LDH) were measured by the p-nitrophenyl phosphate method (Wako Co.), with the GLU kit (Sigma Chemical Co., St Louis, MO) and by the tetrazolium salt method (Wako Co.), respectively. The APH activity is expressed as IU per 2×10⁶ cells, GLU activity is expressed as IU per 4×10⁵ cells and LDH activity is expressed as IU per 1×10⁵ cells.

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Production of IL-2, IFNγ and IL-4
Spleen cell suspension (100 µl) at a concentration of 4×10⁵ cells/ml was incubated at 37°C with 5% CO2 in humidified air for 48 h with Con A (5 µg/ml). IL-2, IFNγ and IL-4 activities in culture supernatants were determined using an ELISA kit (Endogen). The samples were frozen and stored at −80°C until use.
Immunomodulation by citrus auraptene

Fig. 1. Effect of auraptene on glucose consumption capacity of peritoneal macrophages from mice cultured for 24–72 h. Values are means ± SE. Statistically significant difference from the control at *P < 0.05, **P < 0.01 and ***P < 0.001.

Statistical analysis
Results are presented as the means ± SE. All measurements were compared by Student’s *t*-test for unpaired samples. Differences with a *P* value <0.05 were considered significant.

Results

Body weight gain and food intake
The body weights of the control mice were 19.0 ± 0.2 g at 8 weeks of age and 20.1 ± 0.3 g at 10 weeks of age. The food intake of the control mice was 2.86 ± 0.1 g/day/mouse. Auraptene administration scarcely affected body weight gain and food intake (data not shown).

Glucose consumption capacity
The results on glucose consumption capacity in peritoneal macrophages are shown in Figure 1. In 24 h cultures, glucose consumption of peritoneal macrophages in mice gavaged with auraptene at dose levels of 100 and 400 mg/kg were significantly higher than that in the control group (*P < 0.05 and *P < 0.01, respectively). Glucose consumption of peritoneal macrophages from mice treated with auraptene at all doses and incubated for 48 and 72 h were significantly greater than that in the control group (*P < 0.05–0.001).

APH, GLU and LDH activities
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APH, GLU and LDH assays are illustrated in Figure 2. APH activity of the peritoneal macrophages in the auraptene-treated mice at a dose of 100 mg/kg was significantly higher than that in the control group (*P < 0.001). However, doses of 200 and 400 mg/kg slightly increased APH activity. GLU activity of peritoneal macrophages in the auraptene-treated mice at all doses was significantly higher than that in the control group (*P < 0.001). However, there were no significant differences in the LDH activity of peritoneal macrophages among all groups.

Production of IL-1β and TNFα
The results on IL-1β and TNFα production are illustrated in Figure 3. Auraptene at any dose did not enhance spontaneous IL-1β and TNFα production by unstimulated peritoneal macrophages. IL-1β production of peritoneal macrophages stimulated by LPS in mice treated with auraptene at all doses were significantly higher than that in the control group (*P < 0.05–0.001). TNFα production of peritoneal macrophages stimulated by LPS in mice given auraptene at a dose of 200 mg/kg was significantly higher than that of the control group (*P < 0.05).
Splenic lymphocyte proliferative responses
Auraptene did not enhance spontaneous splenic lymphocyte proliferation in mice at any dose. The results on Con A-stimulated splenocyte proliferative responses are shown in Figure 4. SI in mice gavaged with auraptene at a dose of 200 mg/kg were significantly higher than that in the control group ($P < 0.05$).

Production of IL-$2$ and IFN-$\gamma$
The results of assays for IL-$2$ and IFN-$\gamma$ are indicated in Figure 5. When splenic cells were cultured without Con A, IL-$2$ and IFN-$\gamma$ were not detectable in the supernatant. However, cells cultured with Con A produced significant amounts of IL-$2$ and IFN-$\gamma$. The amounts of IL-$2$ and IFN-$\gamma$ produced on stimulation by Con A in mice given auraptene at dose levels of 100 and 200 mg/kg were significantly higher when compared with that in the control group ($P < 0.05$).

IL-$4$ production
The data on IL-$4$ assay are shown in Figure 6. As in the case of IL-$2$ and IFN-$\gamma$ production in splenic cells, auraptene treatment at any dose did not enhance spontaneous IL-$4$ production by splenocytes in mice. There were no significant differences in the IL-$4$ production of splenic lymphocytes stimulated by Con A in mice among all groups.

Discussion
The results in the current study clearly indicate that oral administration of auraptene effectively enhances both macrophage and lymphocyte functions in mice. A recent study suggested involvement of the immune response in chemically induced carcinogenesis (38). In that study, the number of immune cells positive for dendritic cell and macrophage common markers was significantly greater in a $N$-methyl-$N'$-nitro-$N$-nitrosoguanidine-resistant rat strain (Buffalo) than that in a carcinogen-sensitive rat strain (ACI). Thus, the findings in this study suggest that immunomodulation by auraptene may be partially responsible for inhibiting chemically induced carcinogenesis.

Macrophages are known to play an important role in host defense mechanisms for protection from microbial invaders (reviewed in ref. 39). Macrophage glucose consumption increases as a result of activation of macrophages (40). In the present study, glucose consumption capacity of peritoneal macrophages from mice treated with auraptene at all doses incubated for up to 72 h was significantly higher compared with the control group, except for 200 mg/kg and 24 h incubation. These results suggest that auraptene activates peritoneal macrophages. Since glucose consumption by macrophages is related to the pentose phosphate pathway of glycolysis (40), it is likely that auraptene activates the pentose phosphate pathway in peritoneal macrophages.

It is known that activities of lysosomal and cytoplasmic enzymes in peritoneal macrophages increase after activation (41,42). In this study, we evaluated the function of the elimination stage of the phagocytic process by measuring the lysosomal enzymes APH and GLU and the cytoplasmic enzyme LDH in peritoneal macrophages from mice treated with auraptene. APH activity of peritoneal macrophages from the mice given auraptene at a dose of 100 mg/kg and GLU activity of peritoneal macrophages from mice treated with auraptene at all doses were significantly higher than that in the control group. On the other hand, there were no significant differences in LDH activity of peritoneal macrophages in all groups. These results suggest that auraptene activates macrophages through modulation of the activities of the
lysozyme enzymes APH and GLU and also that auraptene partially affects the ability of lysosomal enzymes in peritoneal macrophages to respond appropriately to foreign substances. In the present study, however, auraptene administration did not affect LDH activity in peritoneal macrophages. APH, GLU and LDH activities are stimulated by different mechanisms in vivo, as reported by Allison et al. (41). The finding that auraptene did not affect the LDH activity of peritoneal macrophages suggests that a difference in susceptibility of each enzyme to auraptene stimulation exists. Administration of auraptene at a dose of 100 mg/kg significantly elevated APH activity of peritoneal macrophages but exposure to auraptene at doses of 200 and 400 mg/kg did not. The reason for this is unknown. A lower dose (50 mg/kg) of auraptene in a preliminary experiment increased APH activity (2.10 ± 0.38 IU). These data, therefore, may suggest that the optimal dose of auraptene for elevation of APH activity of peritoneal macrophages is <100 mg/kg.

When macrophages are stimulated with foreign substances, a variety of cytokines and chemicals are released to induce fundamental defense systems. Among them, IL-1 and TNFα are representative cytokines secreted by macrophages that play a key role in the cytokine network, e.g., T cell and NK cell activation (reviewed in ref. 39). Also, IL-1 and TNFα are major pro-inflammatory cytokines characteristically produced at sites of inflammation by macrophages (43) and IL-1 and TNFα are considered to help in eliminating certain invaders (44). In this study, administration of auraptene significantly increased IL-1β production by peritoneal macrophages stimulated by LPS. Auraptene exposure at a dose of 200 mg/kg enhanced TNFα production of peritoneal macrophages stimulated by LPS. These results may suggest that auraptene prevents the effects of certain foreign agents, including carcinogens, through enhancement of secretion of cytokines such as IL-1β and TNFα by peritoneal macrophages.

Lectins are known to possess mitogenic activity after binding to lectin receptors (45). T lymphocyte mitogens such as Con A are thought to act through several subsequent steps, initially inducing IL-1 secretion in macrophages. Also, it is known that mitogenic activity, which reflects an early stage in the immune response, has been measured as a first screening of immuno-modulatory activity (46,47). As shown in the present experiment, auraptene at a dose of 200 mg/kg exerted an augmentative effect on spleen cell proliferative responses to Con A. Auraptene is a naturally occurring coumarin-related compound (4,5). Coumarin derivatives have been reported to have enhancing effects on lymphocyte mitogen responsiveness (48). Therefore, the results in the present study suggest that the mitogenic activity of auraptene might be due to the coumarin structure, and 200 mg/kg/day might be an appropriate oral dose of auraptene to enhance lymphocyte responsiveness.

It is accepted that cytokines are major factors involved in regulation of the immune response to antigens and infectious agents. Recently, Th cells were divided into Th1 and Th2 cells from the profiles of cytokine secretion (reviewed in ref. 49). It is known that Th1 cells are able to produce IL-2 and IFNγ, whereas Th2 cells can produce IL-4 and IL-10. Th1 cells up-regulate mainly cell-mediated immunity and down-regulate humoral immunity, whereas Th2 cells act oppositely (50). A Th1/Th2 imbalance is found in cancer patients (51–53). In the current study, auraptene enhanced the production of IL-2 and IFNγ, but not IL-4 production, at doses of 100 and 200 mg/kg. Our results suggest that oral administration of auraptene may affect the production of cytokines from Th1 cells, such as IL-2 and IFNγ, in mice.

In conclusion, the results described here may support the hypothesis that auraptene directly activates macrophage activities, whereas it only primes lymphocytes to display a greater immune response following interaction of splenic lymphocytes with another stimulus, such as Con A. Our findings suggest that auraptene may exert a part of its cancer chemopreventive activity through enhancement of immune function.

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