Anti-metastatic and Immunomodulating Properties of the Water Extract from *Celosia argentea* Seeds

Yoshihiro Hayakawa, Hideki Furi, Koji Hase, Yasuharu Ohnishi, Rieko Sakukawa, Shigetoshi Kadota, Tsuneo Namba, and Ikuo Saiki

Department of Pathogenic Biochemistry, Natural Products Chemistry, and Natural Drug Resources; Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930–0194, Japan.

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We have investigated the anti-metastatic effect of *Celosia argentea* seed extracts (CAE), which have traditionally been used as a therapeutic drug for eye and hepatic diseases in China and Japan. Intraperitoneal (i.p.) administration of CAE for 7 d before tumor inoculation significantly inhibited liver metastasis caused by intraperitoneal injection of colon 26-L5 carcinoma cells in a dose-dependent manner. CAE also showed concentration-dependent mitogenic activity on BALB/c whole splenocytes, whereas incubation of the non-adherent fraction of splenocytes with CAE did not induce this activity. CAE has the ability to induce interleukin (IL)-12 production from macrophages in vitro. Following i.p. administration of CAE the maximal levels of IL-12 and interferon (IFN)-γ production in serum were achieved at 2–3 and 6 h, respectively. Experiments using macrophage- or NK cell-deficient mice revealed that CAE-induced IL-12 in serum was not mediated by macrophages and that IFN-γ production was mainly dependent on natural killer (NK) cells. Since CAE was inactive when the contributions of macrophages were removed in our system, its inhibitory mechanism is likely to be mainly associated with the activation of macrophages to an anti-metastatic state rather than NK cells. CAE administration resulted in increased production of IL-2, IFN-γ and decreased production of a Th2 cytokine (IL-4) from splenocytes stimulated by PMA and A23187. Thus, the anti-metastatic effect by CAE is based on its immunomodulating properties including induction of cytokines such as IL-12, IL-2 and IFN-γ leading to a Th1 dominant immune state and activating macrophages to the tumoricidal state. This may provide a basis for the inhibition of cancer metastasis.

**Key words** *Celosia argentea*. L.; liver metastasis; IL-12, macrophage; NK cell; helper T (Th) cell

Metastasis is one of the major causes of mortality in cancer. From a clinical point of view, the liver is the most common target of the hematogeneous metastasis of digestive system cancers, especially colon cancer, and the prognosis for cases with liver metastasis is extremely bad. If occult micrometastasis that was established at the time of surgery was inhibited, then the prognosis of patients with colon carcinoma would improve.

Murine colon 26 carcinoma cells are an established experimental model of metastasis in BALB/c mice. We have characterized a liver-metastatic variant (colon 26-L5) of the colon 26 carcinoma by an *in vivo* selection method. Colon 26-L5 cells predominantly metastasize in the liver after inoculation *via* the portal vein of BALB/c mice. This model has provided us with an opportunity for evaluating the efficacy of cancer treatment for liver metastasis, especially for occult micrometastases.

The seed of *Celosia argentea* (Amaranthaceae) has traditionally been used as a therapeutic drug for eye and hepatic diseases in China and Japan. Recently, we have reported that the water extract of the *Celosia argentea* seed was an acidic heteroglycan and possessed a significant hepatoprotective effect in both chemically and immunologically induced liver injury models. It was also found to have immunomodulating properties such as the induction of tumor necrosis factor-alpha (TNF-α) in mice, and the production of interleukin-1 beta (IL-1β) and nitric oxide (NO) in a macrophage-like cell line *in vitro*. In the present study, we investigated the effect of *Celosia argentea* extract (CAE) on the experimental liver metastasis of murine colon 26-L5 cells and clarified its anti-metastatic mechanism based on its immunomodulating activity.

**Materials and Methods**

**Preparation of Celosia argentea Extract (CAE)** The dried and pulverized seeds of *Celosia argentea* (Matsura Yakugyo Co., Ltd., Nagoya, Japan) were macerated with distilled water (8 × 2) for 3 h at 95 °C, and then centrifuged at 4000 rpm for 15 min. The supernatant was filtered with paper (Advantec type 2). The total filtrate was concentrated to a small volume in vacuo below 40 °C and ultrafiltrated (Spectra/Port 6 Membrane; Wako Pure Chemicals, Osaka, Japan) in running water overnight. The fraction (molecular weight: >1.0×10^5) was evaporated, lyophilized, and then used as CAE. This fraction contains an acidic heteroglycan which is the active component for its hepatoprotective activity. CAE was free of endotoxins as determined by the Limulus Test (Wako (<0.03 EU/ml) (Wako Pure Chemical Industry, Osaka, Japan).

**Animals** Specific pathogen-free female BALB/c mice, 6 to 8 weeks old, were purchased from Japan SLC, Hamamatsu. The mice were maintained in the Laboratory for Animal Experiments, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University, under laminar-air-flow conditions. This study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

**Cells** The liver metastatic cell line of the colon 26 carcinoma (colon 26-L5) was obtained by the *in vivo* selection method of Fidler. Colon 26-L5 cells were maintained as monolayer cultures in RPMI-1640 supplemented with 7.5% fetal bovine serum (FBS) and 1-glutamine.

**Assay for Experimental Liver Metastasis of Tumor Cells**

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Log-phase cell cultures of colon 26-L5 cells were harvested with 1 mM EDTA in phosphate-buffered saline (PBS), washed with serum-free RPMI-1640, and resuspended at appropriate concentrations in this medium. BALB/c mice under ether anesthesia underwent laparotomy by an upper median incision, and the duodenal loop was exposed. An injection of colon 26-L5 (1-2×10^5/200 μl) cells was given into the portal vein through a 29-gauge needle attached to a 1-ml syringe. A sterile absorbable cotton sponge was placed over the injection site as the needle was withdrawn to prevent bleeding and peritoneal dissemination of the tumor cells. The mice were sacrificed 19 d after tumor inoculation and the liver weight was recorded to evaluate the tumor metastasis as previously described. CAE was administered i.p. to mice at appropriate doses for 7 d before tumor inoculation.

Preparation of Mouse Splenocytes and Peritoneal Exudate Cells Splenocytes were obtained by passing through a stainless mesh, and treatment with a hypotonic solution to lyse erythrocytes, before being washed three times. The viability was more than 95%, as assessed by the trypan blue dye exclusion method. Unfractionated whole splenocytes suspended in 10% FBS-RPMI-1640 medium (1×10^7/ml) were seeded onto 60×15 mm tissue culture plastic dishes. After a 1 h incubation at 37°C, non-adherent splenocytes were collected by gently washing 3 times with prewarmed PBS and then resuspended in RPMI-1640 medium. T cell-rich splenocytes were obtained by passing the non-adherent splenocytes through a lightly packed nylon wool column. The recovered cells were washed by centrifugation at 1500 rpm for 10 min, and resuspended in RPMI-1640 medium. The peritoneal exudate cells were collected by peritoneal lavage 4 d after i.p. injection with 2 ml of thioglycolate broth, and resuspended in complete RPMI-1640. The macrophage population was prepared by the plastic adherence procedure. The exudate cells (10^6 cells/well) in 24-well culture plates were incubated for 1 h in a 5% CO₂ incubator, and non-adherent cells were removed by vigorous washing with PBS. The remaining adherent cells were used as peritoneal macrophages (PEMs).

Splenocyte Proliferation Assay in Vitro Splenocytes (1-4×10^5) suspended in RPMI-1640 medium supplemented with 10% FBS were cultured in 96-well culture plates with various concentrations (0.1-1000 μg/ml) of CAE for 3 or 5 d at 37°C. The WST solution (WST-1 Cell Counting Kit; Wako Pure Chemicals, Osaka, Japan) was added to a final concentration of 10% 4 h before the termination of the culture. The absorbance of the culture was measured at 450 nm in an immuno-reader (Immuno Mini NJ-2300, Nippon InterMed K.K., Tokyo). Lipopolysaccharide (LPS; E. coli 055:B5, Difco Laboratories, Detroit, MI) and Concanavalin A (Con A; Seikagaku Kogyo Co., Ltd., Tokyo) were used as mitogens.

Induction of Cytokine Production To measure the cytokine level in serum, blood was collected from mice given an i.p. injection of CAE (4 mg/mouse) at 1, 2, 3, 4, 6, and 12 h. Serum was stored at -80°C until the ELISA assay. For the experiment of in vitro cytokine production from macrophages, PEMs in 24-well culture plates were then cultured with or without CAE (10-1000 μg/ml) for 24, 48 h or 7 d at 37°C. Cell-free supernatants were collected and stored at -80°C until the ELISA assay. For the experiment of in vitro cytokine production from splenocytes, CAE (4 mg/mouse) was administered i.p. to mice for 7 consecutive days before the harvest of splenocytes. The T cell-rich splenocytes (5×10^6/well) in 24-well culture plates were then cultured with or without 1 μM phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, U.S.A.) and 2 mM calcium ionophore A23187 (Sigma, St. Louis, U.S.A.) for 24 or 48 h at 37°C. Cell-free supernatants were collected and stored at -80°C until the ELISA assay. Various cytokines in serum and the culture supernatant of splenocytes or macrophages were determined by ELISA kits according to the manufacturer's procedures; mouse IL-12, IL-2, IL-4 and IL-10 (Biosource, Camarillo, CA, U.S.A.), and mouse IFN-γ (Endogen, Boston, MA, U.S.A.).

Statistical Analysis Statistical significance of differences between the groups was determined by the Student's two-tailed t-test.

RESULTS

Inhibition of Experimental Liver Metastasis by CAE We first examined the effect of i.p. administration of CAE on liver metastasis caused by the injection of colon 26-L5 carcinoma cells into the portal vein. The liver weights were measured on day 19 after tumor inoculation. Figure 1 shows that the i.p. administration of CAE for 7 consecutive days before tumor inoculation significantly attenuated the increase of the liver weight in a dose-dependent manner ranging from 0.1 to 4 mg/d. The administration of CAE did not show any adverse effects such as a decrease in body weight (data not shown). These results clearly indicate that CAE prevents the experimental liver metastasis caused by the colon 26-L5 cells.

Effect of CAE on the Proliferation of Splenocytes in Vitro To clarify the biological properties of CAE, we investigated the mitogenic activity of CAE in vitro. Splenocytes were co-cultured with or without various concentrations of CAE for 3 or 5 d. CAE showed mitogenic activity on whole spleen cells in a concentration-dependent manner ranging from 0.1-1000 μg/ml (Fig. 2). LPS and Con A as positive controls were also mitogenic in whole spleen cells. When
CAE was cultured with non-adherent splenocytes (Fig. 3), it showed no mitogenic activity. These results indicate that the expression of mitogenic activity of CAE in whole splenocytes may be associated with the adherent splenocytes (presumably macrophage/monocyte lineage cells) or their derived cytokines.

**Effect of CAE on IL-12 Production from Macrophages**

IL-12, produced by macrophages and other antigen-presenting cells, is widely known to have potent anti-tumor and anti-metastatic activities. We therefore investigated the effect of CAE on the production of IL-12 from macrophages. PEMs were cultured with or without CAE (10–1000 µg/ml) for 24, 48 h or 7 d. As shown in Table 1, the macrophages exposed to CAE were able to produce a high amount of IL-12 into the culture supernatant in a concentration- and time-dependent manner, whereas untreated macrophages did not produce any detectable amount of IL-12 (<2 pg/ml). This result indicates that CAE has the ability to induce IL-12 production from macrophages in vitro.

**Cytokine Production in Vivo**

We next examined whether or not the administration of CAE can induce the production of cytokines such as IL-12 and IFN-γ in vivo. The cytokine level in serum was determined at various time periods after 4 mg of CAE was administered i.p. Figure 4 shows that the IL-12 production in serum increased to a maximum level (about 2000 pg/ml) at approximately 2–3 h after the administration and thereafter gradually decreased. On the other hand, the production of IFN-γ showed its peak response 6 h after the administration.

**Effect of Anti-asialo GM1 Serum and 2-Chloroadenosine on Cytokine Production in Vivo**

The above results demonstrated that CAE had the ability to stimulate macrophages to produce IL-12 in vitro, and also to induce the production of IL-12 and IFN-γ in vivo. Since IL-12 is well known to activate NK cells and subsequently induce IFN-γ production, we next investigated whether or not macrophages or NK cells can be involved in the induction of cytokine production by CAE administration. Anti-asialo GM1 serum can selectively deplete NK cells and 2-chloroadenosine can suppress macrophage function. Figure 4 shows that treatment with 2-chloroadenosine 24 h before CAE administration showed a similar pattern for the production of IL-12 and IFN-γ in serum as compared to untreated mice. In NK cell-depleted mice by treatment with anti-asialo GM1 serum, the level of serum IL-12 after CAE administration was observed almost similarly to that of the untreated mice, but the level of serum IFN-γ 6 h after the CAE administration decreased markedly.

**Effect of Anti-asialo GM1 Serum and 2-Chloroadenosine on CAE-Mediated Inhibition of Liver Metastasis**

Since NK cells and macrophages in the circulation play an important role in the inhibition of tumor metastasis, we next examined whether or not macrophages or NK cells can contribute to inhibition of tumor metastasis by the administration of CAE. 2-Chloroadenosine (50 µg/mouse, Research Biochemicals Incorporated, Natick, MA) or Rabbit anti-asialo GM1 antiserum (200 µg/mouse, Wako Pure Chemical Industry, Osaka, Japan) was administered i.v. at 8 d before (i.e. 24 h before the first administration of CAE) or 24 h before the first administration of CAE.
fore tumor inoculation (i.e., 24 h before the first administration of CAE) or 24 h before tumor inoculation (i.e., immediately after the last administration of CAE). Figures 5 and 6 show that treatment with 2-chloroadenosine or anti-asialo GM1 antisera before tumor inoculation enhanced the frequency of liver metastasis compared to untreated normal mice. CAE did not inhibit liver metastasis in the macrophage-suppressed mice which had been treated with 2-chloroadenosine immediately after the last administration of CAE. CAE administration tended to suppress the increase of liver weight in mice treated with 2-chloroadenosine before CAE administration (i.e., 8 d before tumor inoculation), but this effect was not statistically significant. In contrast, CAE administration for 7 d before tumor inoculation attenuated the increase of the liver weight in NK cell-depleted mice, as well as in normal mice. These results suggest that the inhibition of liver metastasis is partly mediated by the function of macrophages.

**Th1 and Th2 Cytokine Production in Vitro**  IL-12 has multiple immunoregulatory functions including activation of the Th1 subset, which plays a pivotal role in the induction of in vivo anti-tumor immunity. The balance of Th1 and Th2 cells in the host is considered to be important for the regulation and induction of immune functions. We therefore investigated the effect of CAE on the production of Th1- and Th2-type cytokines from splenocytes of CAE-treated mice. T cell-rich splenocytes were obtained from mice given i.p. administration of CAE for 7 d. As shown in Table 2, splenocytes from untreated normal mice and CAE-treated mice did not produce detectable amounts of Th1 cytokines (IL-2 and IFN-γ) or Th2 cytokines (IL-4 and IL-10). When splenocytes were incubated with PMA and A23187 for 24 or 48 h, detectable amounts of these cytokines were induced in the cell-free supernatant. The administration of CAE resulted in a significant enhancement of IL-2 and IFN-γ production as compared with untreated control. In contrast, the production of IL-4 from CAE-treated splenocytes significantly decreased as compared with the untreated control. However, there was no discernible difference in the production of IL-10 between normal and CAE-treated splenocytes co-incubated with PMA and A23187. Thus, administration of CAE may induce Th1-dominant responses in mice.

**DISCUSSION**

Our previous studies have reported that the water extract...
of *Celosia argentea* seeds has hepatoprotective effects in chemical and immunological liver injuries induced by CCl₄, d-Galactosamine (d-GalN)/LPS, Propionibacterium acnes/LPS and d-GalN/TNF-α in mice. However, to our knowledge, the inhibitory effect of the water extract of *Celosia argentea* (CAE) on liver metastasis and its immunomodulatory properties has not been reported.

Intraportal administration of CAE for 7 d before intraperitoneal injection of colon 26-L5 cells resulted in significant inhibition of liver metastasis in a dose-dependent manner (Fig. 1). CAE did not show any side effects nor did it directly affect the growth of the colon 26-L5 cells at concentrations ranging from 0.1—1000 µg/ml in vitro (data not shown). Moreover, CAE (0.1—1000 µg/ml) did not inhibit tumor cell invasion to the reconstituted basement membrane, which is considered to be a crucial step in the complex multistage process of metastasis (data not shown). These findings clearly indicate that CAE is effective for the prevention of liver metastasis, but this anti-metastatic effect is not based on the direct inhibition of tumor cell growth and invasion.

On the other hand, CAE showed mitogenic activity in a concentration-dependent manner (Fig. 2), and this activity depends on the adherent fraction of splenocytes (Fig. 3). Cytokines produced by adherent splenocytes may be necessary for the immunomodulatory activity of CAE in mouse splenocytes. Our previous study has also shown that the water extract of *Celosia argentea* seeds has the ability to produce TNF-α in vivo and activates 3774.1 macrophage-like cell lines to produce IL-β and NO in vitro. The cytokine levels of the macrophage/monocyte lineage cells are known to be altered in several diseases including neoplasia.

IL-12 is a heterodimeric molecule produced by macrophages and other APCs early in the immune response. This cytokine has the ability to induce IFN-γ production by T and NK cells, to enhance cytolytic responses, and has potent antitumor and antimetastatic activity. As shown in Table 1, when CAE was incubated with PEMS, it was able to induce the production of IL-12 in vitro. Similarly, on i.p. administration of CAE, maximum levels of IL-12 and IFN-γ production in serum were achieved at 2—3 and 6 h, respectively (Fig. 4). These findings clearly indicate CAE has an ability to induce IL-12 production in vitro and in vivo. The pattern of IL-12 and IFN-γ production in serum of 2-chloroadenosine-treated mice after CAE administration was almost similar to that in untreated normal mice (Fig. 4). This finding suggests that CAE-induced IL-12 production in vivo is mediated by other IL-12 producing cells such as dendritic cells, monocytes and neutrophils, although CAE had an ability to induce IL-12 production from macrophages in vitro (Table 1). In contrast, a peak production of IFN-γ in serum after CAE administration was not observed in anti-asialo GM1 serum-treated mice (Fig. 4). This suggests that production of IFN-γ by CAE administration is mainly mediated by NK cells. Considering the time difference in the peak responses for the production of these cytokines, the production of IFN-γ in serum may be induced by the IL-12 produced by the administration of CAE.

Since metastasizing tumor cells interact with immune cells which are important in the destruction of tumor cells, we investigated whether CAE can stimulate immune cells to induce the inhibition of tumor metastasis. Figures 5 and 6 show that liver metastasis was enhanced in mice pretreated with 2-chloroadenosine or anti-asialo GM1 serum 24 h or 8 d before tumor inoculation, compared to untreated normal mice. This indicates that macrophages and NK cells have an important role in the prevention of the metastatic spread of colon 26-L5 cells. CAE did not inhibit the liver metastasis of colon 26-L5 cells in mice pretreated with 2-chloroadenosine 24 h before tumor inoculation, whereas it tended to inhibit the metastasis in the mice pretreated with 2-chloroadenosine 8 d before tumor inoculation. It has been also reported that macrophages surrounding regressing tumors were activated by IL-12 and were necessary for the induction of IL-12-mediated anti-tumor effect. Thus, CAE administration may result in the induction of anti-metastatic macrophages rather than IL-12 production. In contrast, CAE was able to inhibit liver metastasis in mice pretreated with anti-asialo GM1 serum, but it did not completely inhibit the metastasis as compared to untreated normal mice. This indicates that CAE-mediated partial inhibition of tumor metastasis in NK cell-depleted mice may depend on another immune effector cells such as macrophages, T cells and NKT cells other than NK cells. Since CAE was inactive when the contributions of macrophages were removed in our system, its inhibitory mechanism is likely to be associated with the activation of macrophages. However, NK cells involved in CAE-mediated inhibition of liver metastasis, because CAE did not show complete suppression of the metastasis in NK cell-depleted mice.

Despite the importance of macrophage- and NK cell-mediated anti-metastatic activity by CAE, the evidence points to the involvement of T cells for the inhibition of metastasis. The balance of helper T cell subsets (Th1-Th2 balance) in the host is considered to be important for the regulation and induction of immune functions. It is now widely recognized that IL-12 has several biological actions including play-
ing a pivotal role in the initiation of cell-mediated immunity via regulation of Th1 and Th2 subsets.25–37 Cancer patients and tumor-bearing animals have been reported to have a marked decrease in Th1 dominant cellular immunity including delayed-type hypersensitivity, cytotoxic functions, and NK cell activity.38,39 Consequently, the increased level of antibody in tumor-bearing animals was observed based on Th2 dominance, thus indicating that the antibody may mask the tumor antigens and prevent an effective anti-tumor cellular response. Therefore, the critical balance of Th1 and Th2-type cytokines such as IL-2, IFN-γ, IL-4 and IL-10 is essential for determining the immune response.22,23 Administration of CAE resulted in the increased production of IL-2 and IFN-γ, and decreased production of IL-4 from splenocytes stimulated by PMA and A23187 (Table 3), thus leading to the Th1 dominant immune state in mice. These results may be associated with the ability of CAE to induce IL-12 production. It has also been reported that i.p. administration of CAE suppressed the formation of IgE antibody in mice which is thought to depend mainly on Th2-type cytokines.40 Therefore CAE is sure to lead to a Th1 dominant state before tumor inoculation and ensuring effective cellular immunity in tumor-bearing mice.

In conclusion, we have demonstrated the possibility that CAE may be an attractive agent to prevent liver metastasis; this is the first evidence that an acidic heterocyclic, including CAE, can inhibit tumor metastasis. The anti-metastatic effect of CAE is associated with its immunomodulating properties including induction of IL-12 production leading to a Th1 dominant state and activation of macrophages, but the detailed mechanism of its anti-metastatic and immunomodulating effects is not fully understood. Further study will be required to determine these points in detail.

REFERENCES