NOTE *Immunology*

Antitumor Effect of Humus Extract on Murine Transplantable L1210 Leukemia

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ABSTRACT. Humic substances are formed during the decomposition of organic matter in humus that found in many natural environments in which organic materials and microorganisms have been present. In the present study, humus extract exhibited antitumor effect on L1210 tumor development in isogeneic DBA/2 mice with the delay of tumor formation and a significant smaller tumor mass that infer a significant increase of life span of mice. The antitumor effect was not due to direct killing of L1210 or induction of apoptosis in tumor cells by humus extract.

KEY WORDS: antitumor activity, humic substance, humus extract.

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Humic substances are formed during the decomposition of organic matter in humus. They can be found in many natural environments in which organic materials and microorganisms have been present [13]. Natural humification products such as humus, peat, sapropel and mumie have been used to develop pharmacologic agents with diverse applications in medical practice [5, 12, 14]. These have been successfully used as anti-inflammatory agents because they have local anti-inflammatory and analgesic properties [6, 10, 14]. Antimicrobial effect of coal-derived humic acid and fulvic acid [9] and humus extract [2] have been investigated. The anti-HIV activity of oxihumate [11] and synthetic humic acid analogues [8] has been also reported. However, the potential of humic substances against tumor has scarcely been investigated. Therefore, an *in vivo* antitumor activity of humus extract was studied against transplantable lymphocytic leukemia L1210 cells in mice.

Humus extract was prepared from humus (collected in Nagasaki Prefecture, Kyushu, Japan) according to the method described by Kodama *et al.* [2]. To the humus, 6 volumes of dechlorinated water (v/w) was added and the mixture was agitated every day for 30 days, then left to stand at 25 to 28°C for 4 months. Supernatant was collected and filtered using a membrane filter (pore size: 25 μm). The resulting humus extract had pH2.8 and contains various minerals including Al, Ca, Mg, Na and Si. The extract contained 1,500 ppm of sulfate. No culturable bacteria were found in the extract. There were small amounts of protein and carbohydrate (0.7% of the total weight).

Eight-week-old inbred DBA/2 mice of both sexes were divided in groups (6 mice in each group) and administered humus extract in dechlorinated tap water (3% or 6%) *ad libitum* for 18 days before tumor cells was injected. Control mice were administered water without humus extract. Isogeneic L1210 lymphocytic leukemia cell line cells (gifted by the courtesy of Dr. Kiyomiya, Laboratory of Veterinary Toxicology, Graduate School of Life and Environmental Science, Osaka Prefecture University, Sakai, Japan) were cultivated in Eagle's Minimum Essential Medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10 % fetal bovine serum and antibiotics at 37°C. Each mouse was inoculated 10^6/0.1 m*l* of L1210 cells subcutaneously on the back. Humus extract was administered for 12 consecutive days after tumor cell inoculation. The mice were observed for any formation of tumors on the inoculated site; the experiment was terminated when tumor mass exceeded 18 × 18 mm, according to the guidelines of the Osaka-Prefecture University Committee in Animal Care and Use.

As shown in Fig. 1, gross tumor was first observed 7 days after L1210 cell inoculation in groups administered 6% humus extract and control mice, whereas tumor was formed 9 days after the inoculation in mice administered 3% extract. Tumors developed continuingly thereafter in the three mice groups, but mice treated 3% humus extract elicited a significant slow tumor growth; namely mean tumor mass was significantly smaller when compared to control group (P<0.001–0.01, Student's t test).

In the second trial, mice were administered 3% humus extract for 18 days before tumor cells were injected. Mice were inoculated different numbers of L1210 cells (10^5, 10^4, 10^3 or 10^2 cells/0.1 m*l*/mouse). Humus extract was administered throughout the experimental period. Figure 2 shows that humus extract suppressed the tumor growth in mice regardless of the number of tumor cells inoculated. Mean tumor mass was significantly smaller in groups inoculated 10^5, 10^4 and 10^2 cells when compared to those of control mice groups (P<0.001–0.05).

In vitro effect of humus extract on L1210 cell proliferation was examined. L1210 cells (2×10^6 cells/2 m*l* in 24-well culture plate) were cultivated for five days in the presence of 0% to 6% of humus extract. Viable cell number was estimated every day using a hemocytometer by trypan blue dye exclusion test. As shown in Fig. 3, high concentration

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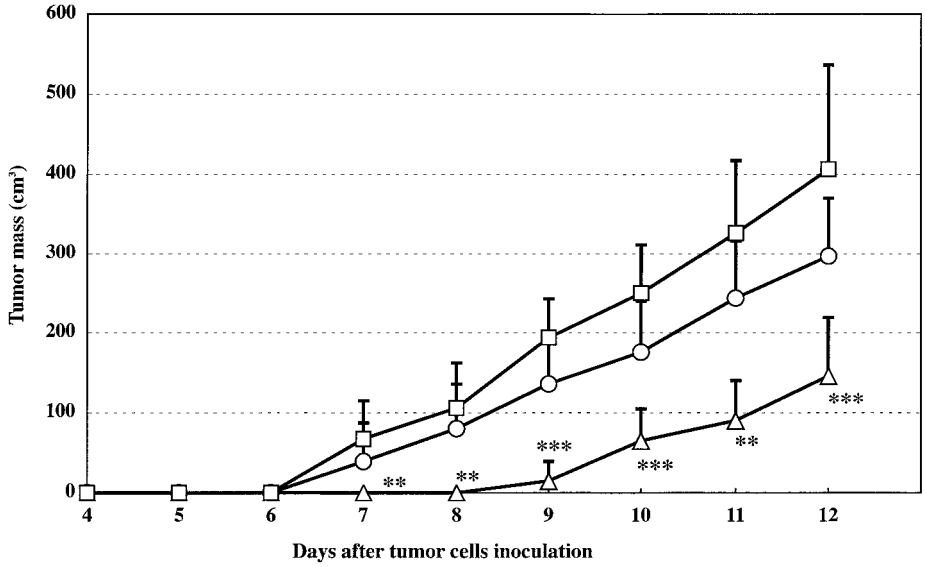

Fig. 1. Suppression of tumor growth in mice administered with 3% (△) or 6% (○) humus extract after inoculation of 10^6/0.1 m*l* of L1210 cells subcutaneously on the back. Statistical significance was determined by Student's *t* test compared to mice administered without humus extract (□) (***; $P<0.001$ or **; 0.01).

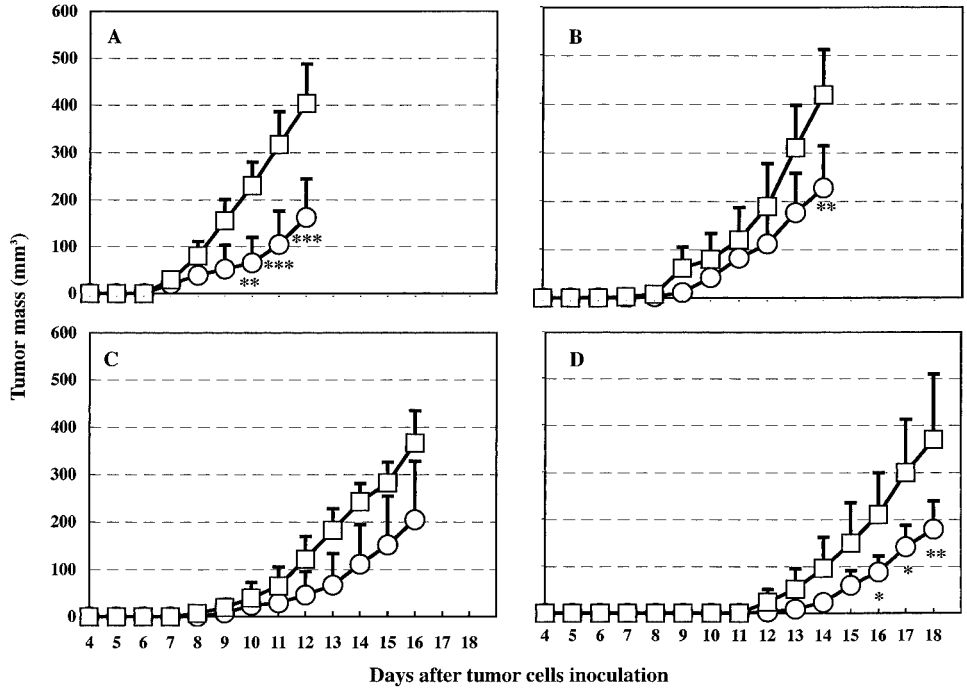

Fig. 2. Suppression of tumor growth in mice administered with 3% (○) humus extract after inoculation of different numbers of L1210 cells (A; 10^5, B; 10^4, C; 10^3 or D; 10^2 cells/0.1 m*l*). Tumor mass was statistically compared to mice administered without humus extract (□) (***; P<0.001, **; 0.01 or *; 0.05).

of humus extract in cultures (3% or 6%) suppressed the growth of L1210 cells, but 0.5% or 1% did not affect the growth of the cells. No significant increase of cells displaying morphological features of apoptosis such as nuclear condensation and the appearance of apoptotic bodies was observed in any group when the cells were stained with Hoechst 33258 (Sigma, St. Louis, MO) and observed under a fluorescence microscope.

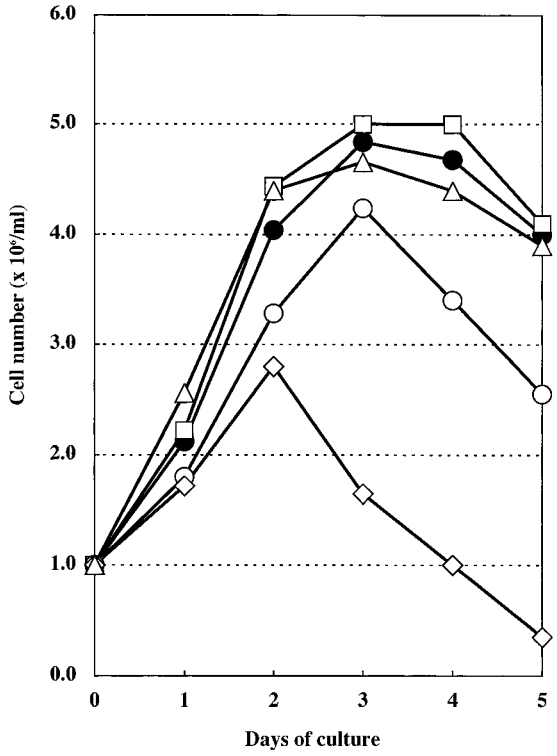

Fig. 3. *In vitro* growth curve of L1210 cells. The cells were cultivated in the presence of 6% (◇), 3% (○), 1% (□), 0.5 % (△) humus extract or without (●) humus extract.

Thus, 3% humus extract exhibited antitumor effect on L1210 tumor development with the delay of tumor formation and a significant smaller tumor mass, indicating that mice administered the extract may survive for longer period than untreated mice after formation of tumors. Tumor growth in mice administered 6% of humus extract, however, was not suppressed significantly compared to control mice, indicating that there exists optimal condition for administration of the extract to animals. Therefore, it is needed to determine optimal concentration and duration of administration of the extract, since we observed similar phenomenon in experimental infections of ulcer disease in fish [2] and trypanosomiasis in mice (unpublished data). The antitumor effect was not due to direct killing of L1210 or induction of apoptotic cell death by humus extract, since *in vitro* test showed no remarkable growth suppression of the cells by the extract (0.5% or 1%) except at high concentrations (3% or 6%). Also, no apoptotic feature was observed in L1210 cultivated in the presence of humus extract. Though the mechanism by which humus extract suppresses the tumor growth is not clear at present, the antitumor activity may involve an enhancement of innate host resistance. Therefore, further studies are required to determine how innate immune responses are induced after administration of humus extract in mice. It is reasonable to assume that humic substances absorbed via the intestinal tract affect host physiological conditions conferring protection against tumor cell growth. Mumie has been reported to enhance [^{3}H]thymidine uptake by mice splenic lymphocytes [7]. Also humic substance enhanced the proliferative capability of thymocytes stimulated by mitogens, and prevented the immuno-suppressive effect of hydrocortisone [4]. Proliferation of lymphocytes in response to humus extract and purified fulvic acid, and cytotoxic activity of lymphocytes of humus-treated mice against L1210 cells is now under investigation. Since fulvic acid did not cause any apparent adverse effects [5, 10], humic substances and/or humus extract can be used in animals as food additives and for immunopotentiating materials. However, it is recently reported that humic acid is implicated as an etiologic factor in the vasculopathy of blackfoot disease in Taiwan [1], and it induces apoptotic changes in cultured human cells and promotes neoplastic transformation in the cells [3]. Therefore, further analysis of antitumor mechanisms activated by humus extract, and separation of biologically active components in humus, are needed to elucidate the effect and adverse effects of humic substance.

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