Antitumor Activity of Methanol Extract from Roots of *Agrimonia pilosa* LEDEB.

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Abstract—To evaluate the antitumor activity of *Agrimonia pilosa* LEDEB., the effects of the methanol extract from roots of the plant (AP-M) on several transplantable rodent tumors were investigated. AP-M significantly prolonged the life span of S180-, Meth-A fibrosarcoma- and MM-2 mammary carcinoma-bearing mice by intraperitoneal (i.p.) pre- or postmedication. AP-M also inhibited the growth of S-180 solid type tumor. On the other hand, the prolongation of life span induced by AP-M on S-180 ascites type tumor-bearing mice was markedly minimized or abolished by the pretreatment of cyclophosphamide. AP-M showed considerably strong cytotoxicity on MM-2 cells in vitro, but the effect was diminished to one-tenth by the addition of serum to the culture. Against the host animals, the peripheral white blood cells in mice were significantly increased from 2 to 5 days after the i.p. injection of AP-M. On 4th day after the injection of AP-M, the peritoneal exudate cells which possessed the cytotoxic activity on MM-2 cells in vitro were also increased to about 5-fold those in the non-treated control. The spleen of the mice was enlarged, and the spleen cells possessed the capacity to uptake ^3H -thymidine. However, AP-M did not show direct migration activity like other mitogens against spleen cells from non-treated mice. These results indicate that the roots of *Agrimonia pilosa* contain some antitumor constituents, and possible mechanisms of the antitumor activity may be some host-mediated actions and direct cytotoxicity.

Agrimonia pilosa LEDEB. (Rosaceae, Japanese name: Kinmizuhiki) has been used as an antidiarrheic, a hemostatic and an anti-parasitic in Japan and China (1, 2). Furthermore, we saw a description about the effectiveness of the plant on some carcinomas in Ben-cao-gang-mu (3), and the plant is being used for cancer therapy in China today (4). However, there are only a few reports describing fundamental experiments on the antitumor activity of this plant (5, 6).

In the present study, we investigated the antitumor activity of the methanol extract from roots of *Agrimonia pilosa* LEDEB. against some transplantable rodent tumors.

Materials and Methods

Animals and tumors

Female 6-weeks-old ddY, BALB/c and C3H/He mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu. S-180, Meth-A fibrosarcoma and MM-2 mammary carcinoma were maintained by intraperitoneal (i.p.) passage at weekly intervals in ddY, BALB/c and C3H/He mice, respectively.

Preparation of the methanol extract

The roots of *A. pilosa* growing wildy around this university were collected in early autumn. The roots (800 g) were dried and powdered and then extracted three-times

with 4.5 l of methanol in reflux. The methanol solution was cooled, filtered and dried *in vacuo* to give a brown residue (AP-M, 139.7 g). AP-M was dissolved or suspended in phosphate-buffered saline (PBS) immediately before use.

Agents

OK-432 (Chugai), cyclophosphamide (CPA, Shionogi) and concanavalin A (Con A, Maruzen Oil) were commercially purchased. *Klebsiella* 03 lipopolysaccharide K03 LPS) was prepared from the culture supernatant of decapsulated mutant strain LEN-1 (03: K1⁻) derived from *K. pneumoniae* strain Kasuya as described previously (7).

Antitumor experiments in mice

To evaluate the antitumor activities against ascites type tumors, mice were i.p. inoculated with tumor cells (1×10^5 or 10^6 S-180 cells/ddY mouse, 1×10^5 Meth-A cells /BALB/c mouse, 5×10^5 MM-2 cells/C3H/He mouse) on day 0 according to the treatment schedules indicated in the tables. Sixty days after the cell inoculation, survivors were killed and autopsied. For S-180 solid type tumor, mice were subcutaneously (s.c.) inoculated at the left inguinal region with 3×10^6 tumor cells on day 0 and were i.p. medicated on days 1 to 12. Thirty-six days after the cell inoculation, the tumors were removed and weighed.

Assay of direct cytotoxic activity against MM-2 cells

A 2 ml aliquot of tumor cells (2×10^5 cells/ml) prepared in RPMI 1640 medium with or without 10% fetal calf serum was added with graded concentrations of AP-M and was incubated at 37°C for 2 hr. Then, cells were washed with Hanks' solution and were further incubated in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 μ M mercaptoethanol at 37°C in a humidified CO₂ incubator for 48 hr. The viable cells were assayed by a dye exclusion method with 0.2% Trypan Blue.

Assay of number and population of peripheral white blood cell (WBC)

The peripheral blood was obtained from the mice treated with AP-M by the ocular fundus puncture. Total WBC number was estimated by using a Bürker-Türk hemocytometer. For the determination of the cell population, cells were stained with Giemsa

stain, and about 400 nucleated cells were counted for each slide glass.

Harvesting of peritoneal exudate cell (PEC) and cytotoxicity of adherent PEC

PECs were collected from the mouse 4 days after i.p. injection of AP-M or OK-432 by washing the peritoneal cavity with 3 ml of Hanks' solution and were washed 3 times with RPMI 1640 medium. The cell number was estimated by the standard hemocytometer technique. PECs (5×10^6 cells) suspended in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 μ M mercaptoethanol were placed on a 35-mm plastic petri dish and were cultured for 1 hr. The dish was washed to remove non-adherent cells. Then, a 2 ml aliquot of MM-2 cells (5×10^5 cells) suspended in the culture medium was added to the dish and was cultured for 40 hr at 37°C. After the culture, the viable MM-2 cells were counted by the Trypan Blue dye exclusion method.

Experiments of ³H-thymidine (TdR) uptake by spleen cells

1) ³H-TdR uptake by spleen cells from the mice treated with AP-M or OK-432: A single cell suspension was prepared from the spleen of the mouse treated with each agent by the standard technique. A one ml aliquot of the cell suspension (1×10^6 cells/ml of RPMI 1640 medium supplemented with 10% fetal calf serum and 10 μ M mercaptoethanol) was incubated for 1 hr and was further incubated with 1.0 μ Ci ³H-TdR for 24 hr at 37°C.

2) ³H-TdR uptake by spleen cells treated with AP-M or mitogens *in vitro*: A one ml aliquot of the cell suspension (1×10^6 cells) prepared from non-treated mice spleen was incubated with AP-M or a mitogen for 24 hr at 37°C. Then, 1.0 μ Ci ³H-TdR was added to the culture medium, and the culture was further incubated for 24 hr.

After the incubation, cells were harvested on a glass fiber filter (Whatman, GF/C) and then washed with cold PBS, 5% trichloroacetic acid and an ethanol:ether (3:1, v/v) mixture. The radioactivity on the filter was counted in a toluene-based scintillator (PPO 4 g, POPOP 0.1 g, toluene 1 l) by a Beckman LS-230 liquid scintillation counter.

Results

Antitumor activity of AP-M against S-180

in mice: When AP-M was i.p. given to ddY mice i.p. inoculated with 1×10^5 cells on day 0, the extract significantly prolonged the life span of the mice bearing S-180 in each of the medication schedules (Table 1). Even when AP-M was given by a single injection 14 days before the tumor inoculation, a marked tumor growth suppression was observed. Among these medication schedules, a single administration of AP-M on day -4 or on day +1 appeared to be more effective. Table 2 shows the results of the experiments of the activity of AP-M against the mice i.p. inoculated with a large inoculum of the tumor cells (1×10^6

cells), and it shows the influence of CPA on the antitumor activity of the extract. The antitumor activity of AP-M was observed clearly, but it was lower than that against the mice inoculated with 1×10^5 cells. CPA (40 mg/kg) was i.p. injected once a day on days -6 to -2. The prolongation of life span induced by AP-M was cancelled or minimized by CPA, while CPA alone hardly influenced the survival time of the mice bearing S-180 ascites tumor.

Next, the antitumor activity of AP-M against solid type tumor was examined. Mice were s.c. inoculated with 3×10^6 cells and were given AP-M i.p., once a day for 12 days from 24 hr after the cell inoculation. As shown in Fig. 1 and Table 3, 3 and 10 mg/kg of AP-M

Table 1. Antitumor activity of AP-M against S-180 ascites type tumor

Medication schedule	Single dose (mg/kg)	Survival days ^a (mean±S.E.)	%ILS ^b	60-day survivors ^c
on days -7~-1	10	28.3±3.7	146	3
on day -14	100	26.5±6.6	130	2
on day -7	100	26.2±4.0	128	1
on day -4	100	31.7±7.4	176	3
on day -1	100	26.5±3.0	130	2
on day +1	100	34	196	5
on day +4	100	20.0±2.9	74	2
on day +7	100	14.3±2.9	24	3
on days +1, 4, 7	100	34.5±5.1	200	2
on days +1~+7	10	36.3±3.3	216	2

Six ddY mice were i.p. inoculated with 1×10^5 cells on day 0 and were i.p. administered with AP-M once a day according to the indicated schedule. The mean survival time of non-treated control mice was 11.5 days. ^aExcluding 60-day survivors. ^b $[(T-C)/C] \times 100$, T: mean survival days of treated mice, C: mean survival days of non-treated control mice. ^cMacroscopic tumor findings were negative.

Table 2. Pretreatment effect of CPA on the antitumor activity of AP-M against S-180 ascites type tumor

Medication schedule	Single dose (mg/kg)	Survival days ^a (mean±S.E.)		%ILS ^b		60-day survivors ^c	
		without CPA (A)	with CPA (B)	(A)	(B)	(A)	(B)
on day -4	100	23.3±2.6	16.6±2.5	130	64	1	0
	100	16.6±1.0	7.0±0.0	64	-31	0	0
on days +1~+7	30	21.4±2.9	7.3±0.3	112	-28	0	0
	10	14.0±2.3	7.6±1.2	39	-25	0	0

Six mice were i.p. inoculated with 1×10^6 cells on day 0 and were i.p. administered with AP-M once a day according to the indicated schedule. CPA (40 mg/kg) was i.p. given once a day on days -6 to -2. The mean survival time of non-treated control mice and CPA-pretreated mice was 10.1 days and 9.4 days, respectively. ^a Excluding 60-day survivors. ^b Described in footnote ^b of Table 1. ^c Macroscopic tumor findings were negative.

significantly suppressed the tumor growth.

Antitumor activity of AP-M against Meth-A ascites type tumor: BALB/c mice were i.p. inoculated with 1×10^5 Meth-A fibrosarcoma cells on day 0 and AP-M was i.p. administered once a day on days -7 to -2 as the premedication or on days +1 to +7 as the postmedication. AP-M also prolonged the life span of the mice. The premedication of 10 mg/kg of the extract was most effective (Table 4).

Antitumor activity of AP-M against MM-2 ascites type tumor: The antitumor activity of AP-M against the mice bearing MM-2 was compared with that of OK-432 (Table 5). C3H/He mice were i.p. inoculated with 5×10^5 cells on day 0. AP-M was i.p. administered once on day -4 as the premedication because the activity of the extract against S-180 ascites type tumor was most effective when the extract was administered this day (Table 1). For the postmedication, AP-M was i.p. administered 3 times on days +1, +4 and

Fig. 1. Effect of AP-M on the growth of S-180 solid type tumor. Ten ddY mice were s.c. inoculated with 3×10^6 cells/mouse on day 0. AP-M was i.p. administered at arrows at doses of 30 (Δ), 10 ($\square$), 3.0 ($\blacktriangle$), 1.0 ($\blacksquare$) and 0 mg/kg (control, $\circ$).

Table 3. Antitumor activity of AP-M against S-180 solid type tumor

Single dose (mg/kg)	Tumor weight ^a (g) (mean $\pm$ S.E.)	% of inhibition ^b
30	4.92 $\pm$ 1.40	36.4
10	1.77 $\pm$ 0.57	77.1
3	2.29 $\pm$ 0.63	70.4
1	7.46 $\pm$ 2.11	3.6
0	7.75 $\pm$ 0.94	—

Ten ddY mice were s.c. inoculated with 3×10^6 cells on day 0 and were i.p. administered with AP-M once a day for 12 days. ^a Wet weight 36 days after the cell inoculation. ^b [(C-T)/T] $\times$ 100, T: mean weight of tumor in the test group, C: mean weight of tumor in the control group.

Table 4. Antitumor activity of AP-M against Meth-A ascites type tumor

Medication schedule	Single dose (mg/kg)	Survival days ^a (mean $\pm$ S.E.)	%LS ^b	60-day survivors ^c
on days -7~-2	30	14.3 $\pm$ 1.6	-6	0
	10	32.7 $\pm$ 5.0	115	2
	3	20.3 $\pm$ 1.9	34	0
	1	16.5 $\pm$ 1.0	9	0
on days +1~+7	30	23.0 $\pm$ 0.8	51	0
	10	20.5 $\pm$ 1.1	35	0
	3	19.5 $\pm$ 0.7	28	0
	1	20.5 $\pm$ 1.0	35	0

Six BALB/c mice were i.p. inoculated with 1×10^5 cells on day 0 and were i.p. administered with AP-M once a day according to the indicated schedule. The mean survival time of non-treated control mice was 15.2 days. ^aExcluding 60-day survivors. ^bDescribed in footnote ^b of Table 1. ^cMacroscopic tumor findings were negative.

Table 5. Antitumor activity of AP-M and OK-432 against MM-2 ascites type tumor

Agent	Medication schedule	Single dose (mg/kg)	Survival days ^a (mean±S.E.)	%ILS ^b	60-day survivors ^c
AP-M	on day -4	100	33.2±1.0	65	1
		30	29	44	5
		10	24.3±5.5	21	3
		3	18.8±0.7	-6	0
	on days +1, 4, 7	100	toxic	—	—
		30	22.8±0.8	13	2
		10	29.8±6.2	48	1
		3	25.5±2.3	27	0
		1	23.2±2.2	15	1
OK-432	on day -4	100 KE/kg	24.1±0.1	20	1
	on days +1, 4, 7	100	32.4±2.4	61	1
		30	25.8±2.3	28	0
		10	21.0±0.7	4	0

Six C3H/He mice were i.p. inoculated with 5×10^5 cells on day 0 and were i.p. administered with AP-M or OK-432 once a day according to the indicated schedule. The mean survival time of non-treated control mice was 20.1 days. ^a Excluding 60-day survivors. ^b Described in footnote ^b of Table 1. ^c Macroscopic tumor findings were negative.

Fig. 2. Direct cytotoxic activity of AP-M against MM-2 cells. Cells (2×10^5 cells/ml) were treated with graded concentrations of AP-M in the absence (○) or in the presence (●) of 10% fetal calf serum for 2 hr. The treated cells were incubated for 48 hr and the viable cells number was determined. Data are the means of triplicate determinations.

+7. OK-432 was administered according to the same schedules in the case of AP-M. AP-M was also effective on the tumor, and the activity was similar or higher than that of OK-432. Additionally, the activity of AP-M was more potent in the premedication than in the postmedication, similar to its action

against other tumor strains.

Direct cytotoxic activity of AP-M against MM-2 cells in vitro: Figure 2 shows the results of the experiments on the direct cytotoxic activity of AP-M against MM-2 cells. MM-2 cells (2×10^5 cells/ml) were treated with AP-M for 2 hr in the presence

or absence of fetal calf serum. The IC₅₀ of AP-M in the absence of serum was 7.3 $\mu\text{g/ml}$, but the cytotoxicity was significantly decreased by addition of serum in the reaction mixture, and the IC₅₀ was 88.0 $\mu\text{g/ml}$.

Influence of AP-M on the number and the cell population of peripheral WBC: C3H/He mice were i.p. administered AP-M (100 mg/kg). The total number of WBC was significantly increased 2 to 5 days after the injection of AP-M. In the cell population, the percentage of lymphocytes was markedly higher and that of monocytes and basophils was also increased. After 11 days, the total cell number was similar to that of non-treated mice, but the percentage of lymphocytes was still high (Fig. 3).

Increase of PEC by AP-M and cytotoxic activity of adherent PEC: C3H/He mice were i.p. administered AP-M once, 4 days before

the experiment. The number of PEC was markedly increased by the injection of AP-M; and when the extract was given to the mice at a dose of 30 to 100 mg/kg, the number was increased to about 5- to 6-fold that of non-treated mice. Furthermore, PECs having the property to adhere on a plastic dish seemed to be macrophages possessing a cytotoxic activity against MM-2 cells (Fig. 4).

Induction of ³H-TdR uptake capacity of spleen cells by AP-M: The spleens of the mice 4 days after the i.p. injection with AP-M were enlarged and the weight was increased. The spleen cells from the treated mice possessed a strong capacity to uptake ³H-TdR, while the cells from non-treated mice hardly did (Table 6). On the other hand, AP-M did not show direct migration activity against spleen cells from non-treated mice. Mitogens used in this study, however, induced the cells to have ³H-TdR uptake capacity (Table 7).

Discussion

Agrimonia pilosa had been known traditionally as a plant possessing an antitumor effect (3). However, we have seen only a few reports on the direct cytotoxic activity of certain extracts of this plant against tumor cells in an in vitro or an in vitro-in vivo test (5, 6). This work demonstrated the antitumor activity of the methanol extract from roots of *Agrimonia pilosa* LEDEB. (AP-M) against murine allogenic and syngeneic tumors in vivo. An important characteristic of the antitumor activity of AP-M was that

Fig. 3. Hematological findings in mice treated with AP-M. Five C3H/He mice in a group were i.p. administered with AP-M (100 mg/kg); and after the indicated days, the number and population of WBC in peripheral blood were determined. Data are the means of 5 mice. Neutrophils: stippled, basophils: white, monocytes: black, lymphocytes: cross-hatched.

Fig. 4. Change of number of PEC in mice treated with AP-M and cytotoxicity of the adherent PEC on MM-2 cells. Five C3H/He mice in a group were i.p. administered 4 days before the experiments with the indicated doses of AP-M or OK-432. See details in "Materials and Methods". Data for the number of PEC are the means of 5 mice. Data of the cytotoxicity test of adherent PEC are the means of triplicate determinations. NT: not tested.

Table 6. Change of spleen weight and ^3H -TdR uptake activity of the spleen cells from mice treated with AP-M

Agent	Dose (mg/kg)	Wet weight		^3H -TdR uptake	
		(mg) (mean $\pm$ S.E.)	% of control	($\times 10^4$ cpm) (mean $\pm$ S.E.)	% of control
AP-M	100	114.5 $\pm$ 4.1	138	9.52 $\pm$ 0.32	262
	30	167.0 $\pm$ 10.8	202	16.50 $\pm$ 0.79	453
	10	91.5 $\pm$ 6.1	111	5.41 $\pm$ 0.16	149
	3	92.5 $\pm$ 2.5	112	5.35 $\pm$ 0.63	147
OK-432	100	114.0 $\pm$ 4.8	138	8.67 $\pm$ 0.13	238
Control		82.8 $\pm$ 2.5	100	3.64 $\pm$ 0.07	100

Four C3H/He mice were i.p. administered with an agent 4 days before the experiments. Spleens were weighed, and a single spleen cell suspension was prepared. Cells (1×10^6) were incubated with $1.0 \mu\text{Ci}$ ^3H -TdR for 24 hr. Experiments were carried out in triplicate for each spleen.

Table 7. Stimulation of spleen cells from non-treated mice

Agent	Concentration ($\mu\text{g/ml}$)	^3H -TdR uptake	
		($\times 10^4$ cpm) (mean $\pm$ S.E.)	% of control
AP-M	50	1.37 $\pm$ 0.05	150
	10	0.84 $\pm$ 0.05	92
	3	0.93 $\pm$ 0.06	102
	1	1.57 $\pm$ 1.78	173
KO3 LPS	50	6.09 $\pm$ 0.09	668
	10	3.54 $\pm$ 0.04	388
	3	2.77 $\pm$ 0.06	304
	1	2.26 $\pm$ 0.04	247
ConA	50	1.20 $\pm$ 0.02	132
	10	27.71 $\pm$ 1.45	3039
	3	17.50 $\pm$ 1.69	1919
	1	4.49 $\pm$ 0.29	492
Control		0.91 $\pm$ 0.10	100

Single cell suspension was prepared from the spleen of a normal C3H/He mouse. Cells (1×10^6) were incubated with each agent for 24 hr and were further incubated with $1.0 \mu\text{Ci}$ ^3H -TdR for 24 hr. Experiments were carried out in triplicate.

premedication of this agent was more effective than its postmedication. When AP-M was i.p. administered 4 days before the i.p. inoculation of S-180 cells, the activity seemed to be highest; and even in the case of injection 14 days before the cell inoculation, the extract significantly prolonged the life span of the mice. AP-M showed similar or higher activity than OK-432 against MM-2 mammary carcinoma in the same medication schedule.

A streptococcal preparation, OK-432, is known to have effective antitumor and

immune modulatory activities in experimental animals (8–10). So, we performed comparative investigations on the activities of AP-M and OK-432 against lymphocytes and macrophages in mice, which are considered to be involved in the immune responses. Total peripheral WBC number was increased from 2 to 5 days after i.p. injection of AP-M, and especially, lymphocytes and monocytes were increased. Moreover, when AP-M or OK-432 was i.p. injected into mice before 4 days, the number of PEC and the weight of the spleen were significantly increased; and

the PECs and the spleen cells possessed capacities of cytotoxic and self-growing activities, respectively. These activities induced by 30 to 100 mg/kg of AP-M were similar or higher than those by 100 KE/kg of OK-432. AP-M did not show direct migration activity against spleen cells from non-treated mice. On the other hand, the antitumor activity of AP-M on S-180 ascites type tumor-bearing mice was markedly minimized or abolished by the pretreatment of CPA.

From these results, it is thought that the antitumor activity of the methanol extract of *Agrimonia pilosa* LEDEB. may be provided by host-mediated actions so that the extract stimulates macrophages and induces cytotoxic macrophages and the stimulated macrophages activate immune charging and cytotoxic lymphocytes.

Agrimonia pilosa is rich in tannin (11) which generally shows potent astringent action, and most of the effects of this plant on diseases are believed to be based on this constituent. However, it is difficult to explain the antitumor activity of AP-M from only the direct cytotoxic activity because if the effective constituent is tannin, it seems that it would rapidly bind to many components in the host animal. In fact, the direct cytotoxicity of AP-M was quite substantial, but was diminished to about one-tenth by the addition of calf serum to the culture. However, we cannot deny the possibility that tannin contained in this plant may act as an anti-tumor constituent. Further studies on the isolation of the effective constituents from this plant and on mechanisms related to the antitumor activity are in progress.

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