Jpn. J. Cancer Res. 84, 99–103, January 1993

Antitumor Activity of Oenothein B, a Unique Macrocyclic Ellagitannin

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The antitumor effect of oenothein B, a macrocyclic ellagitannin from *Oenothera erythrosepala* Bordas, on rodent tumors was studied. Oenothein B exhibited a strong antitumor activity against MM2 ascites tumors upon intraperitoneal administration to the mice before or after the tumor inoculation. The tannin also inhibited the growth of Meth-A solid type tumor in mice. This antitumor effect of the tannin could not be attributed to its direct cytotoxic action on tumor cells, because the cytotoxicity was very weak in the presence of serum protein. When oenothein B was injected into the peritoneal cavity of mice, peritoneal exudate cells, including cytostatic macrophages, were induced. Furthermore, in the *in vitro* treatment of macrophages from mice and humans, the tannin stimulated release of an interleukin 1 (IL-1)-like activity and IL-1β from the cells. These results suggest that oenothein B exerts its antitumor effect through potentiation of the host-immune defense via activation of macrophages.

Key words: Antitumor effect — Tannin — Oenothein B — Macrophage — Interleukin 1

Several plant tannins and related compounds have been shown to have multiple biological activities.[1] The potency of the activities depends on the structural type of tannins. Tannins are classified into two large groups, condensed and hydrolyzable tannins, and the latter group of tannins is further classified into gallotannins having only galloyl groups, and ellagitannins having hexahydroxydiphenoyl group(s), esterifying the sugar core in the molecule, and others.

We have reported that the condensed tannins and related compounds showed negligible antitumor activity, while ellagitannins among the hydrolyzable tannins had antitumor activity against mouse ascites sarcoma S-180.[2-4] Furthermore, we have indicated that *in vivo* treatment with agrimoniin, an antitumor-active ellagitannin from *Agrimonia pilosa* Ledeb., induced cytotoxic adherent peritoneal exudate cells (PEC) and natural killer cell activity.[5,6] Thus, it is suggested that ellagitannins exhibit their antitumor effect by enhancing the immune response of the host. The present study deals with the antitumor effect of oenothein B, an ellagitannin having a unique macrocyclic structure from *Oenothera erythrosepala*, and its primary action mechanism.

MATERIALS AND METHODS

Materials Oenothein B (Fig. 1) was isolated from *Oenothera erythrosepala* Borbas by a method described elsewhere.[7] A streptococcal preparation (OK-432, Chugai Pharmaceutical Co., Ltd., Tokyo) and *Escherichia coli* lipopolysaccharide (*E. coli* LPS, Wako Pure Chemical, Tokyo) were used as positive reference control agents.

The preparation of oenothein B and all media components used in this study were confirmed to be free of endotoxin by means of the *Limulus* amebocyte lysate assay (Wako Pure Chemical).

Antitumor experiments Mouse tumor cell lines of MM2 mammary carcinoma and Meth-A fibrosarcoma were maintained by intraperitoneal passage at weekly intervals in female C3H/He and BALB/c mice (Nippon SLC, Hamamatsu), respectively. In the experiments with the ascites-type MM2 tumor, cells (5×10^5) were inoculated in the abdominal cavity of six female C3H/He mice in a group on day 0, and the intraperitoneal treatment with oenothein B or OK-432 was done 4 days before (day -4) or 1, 4, and 7 days after the tumor inoculation (days 1, 4, 7). The antitumor effect was evaluated at 60 days after the tumor inoculation in terms of the number of regressors and the percent increase in the life span (%ILS) calculated according to the following equation:

$$\%\mathrm{ILS} = \frac{\mathrm{T}-\mathrm{C}}{\mathrm{C}} \times 100$$

where T and C represent the mean survival days of the treated group and the mean survival days of the vehicle control group, respectively.

In the experiment on solid-type Meth-A tumor, cells (1×10^6 cells) were subcutaneously inoculated at the left

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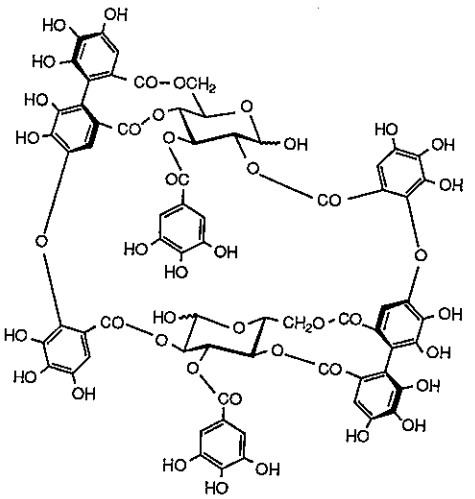

Fig. 1. Structure of oenothein B.

inguinal region of ten female BALB/c mice in a group on day 0, and the intraperitoneal treatment with oenothein B or OK-432 was done on days 8 to 14 once a day. The antitumor effect was evaluated on day 21 in terms of the percent growth inhibition of tumor weight according to the following equation:

$$\% \text{ growth inhibition} = \frac{C-T}{C} \times 100$$

where T and C represent the mean tumor weight of the treated group and the mean tumor weight of the vehicle control group, respectively.

Direct cytotoxicity against MM2 cells A 2 ml aliquot of MM2 cells (2×10^5 cells/ml) prepared in RPMI-1640 medium with or without 10% fetal calf serum was incubated with graded concentrations of oenothein B at 37°C for 2 h. Then, the cells were washed with Hanks' solution and further incubated in RPMI-1640 medium supplemented with 10% fetal calf serum and 10 μM mercaptoethanol (growth medium) at 37°C in a humidified CO_2 incubator for 48 h. The viable cells were assayed by a dye exclusion method with 0.2% Trypan Blue.

Preparation of macrophages The PEC were obtained from C3H/He mice that had received an intraperitoneal injection of thioglycollate, an unstimulating inducer, or oenothein B several days earlier. To obtain macrophages, the PEC were placed in plastic Petri dishes and incubated at 37°C for 1 h in a humidified 5% CO_2 incubator. After removal of nonadherent cells by repeated and intensive washing with the medium, more than 85% of the adherent cells were found to be macrophage-monocytes by Wright-Giemsa staining.[8]

Human source macrophages were obtained from five normal volunteers by Ficol-Hypaque (Lymphoprep™; Nycomed Diagnostics, Oslo, Norway) density gradient centrifugation as described previously.[9]

Cytotoxicity of macrophages Macrophages harvested from control and treated C3H/He mice were suspended in the growth medium, and a 2 ml aliquot (5×10^6 cells) was cultured in each well of a 24-well culture plate for 1 h. Then, MM2 cells (5×10^5) and 3.7 kBq of ^{3}H-thymidine (TdR, New England Nuclear, Boston, MA) were added and cultured for 24 h at 37°C. After the incubation, cells were harvested on a glass fiber filter (Whatman, GF/C) and washed with cold phosphate buffer, 5% trichloroacetic acid, and an ethanol:ether mixture. The radioactivity on the filter was counted in a toluene-based scintillator by using a Beckman LS-230 liquid scintillation counter.

Assay for interleukin 1 (IL-1) Macrophages ($1–2\times10^6$ cells/ml) were treated with or without oenothein B or *E. coli* LPS for 4 h in the growth medium, washed twice, and further cultured, and then the supernatant was collected for assay of IL-1. IL-1-like activity in the supernatant of mouse macrophage culture at 48 h was measured in terms of enhancement of thymocyte proliferation (lymphocytes activating factor (LAF) assay).[10] Thymocytes (1×10^6 cells/well) prepared from C3H/He mice 5 to 7 weeks of age were cultured with 3 μg/ml of concanavalin A (Con A; E.Y. Laboratories Inc., San Mateo, CA) and culture supernatant (50% v/v) of macrophages in 96-well flat-bottomed plates for 66 h, and then pulsed with 18.5 kBq of ^{3}H-TdR for 6 h before harvesting of the cells. The cells were harvested on glass fiber filters and the radioactivity in DNA was measured by liquid scintillation counting. The IL-1-like activity was calculated by use of the following equation: IL-1-like activity = (mean cpm of triplicate assays with a supernatant sample in the presence of Con A) − (mean cpm of triplicate assay with Con A alone).

IL-1β released from human macrophage culture within 24 h was measured by using a commercially available enzyme-linked immunoadherent assay (R&D Systems, Inc., Minneapolis, MN). After coating with a murine antibody against IL-1β, the reactant with IL-1 protein present in the sample supernatant was detected with a second antibody conjugated to horseradish peroxidase, with tetramethylbenzidine and hydrogen peroxide as substrates.

Statistical analysis Significance of differences between the means of treated and control groups was assessed by the use of the chi-square test and Student's t test.

RESULTS

Antitumor activity When oenothein B was intraperitoneally injected into female ddY mice, the 50% lethal dose (LD_{50}) was 80.5 (72.6–90.3) mg/kg as evaluated by a

Litchfield-Wilcoxon method. Subsequently, the maximum single dosage used was 10 mg/kg. As shown in Table I, oenothin B exhibited a dose-dependent antitumor effect on MM2 tumor in C3H/He mice when administered either before or after tumor inoculation, as did OK-432. Tumors appeared to develop in the mice injected with 10 mg/kg of the tannin 4 days before the cell inoculation, but had almost completely regressed by 60 days. Oenothein B also inhibited growth of Meth-A solid tumor, while OK-432 was slightly effective (Table II). On the other hand, oenothein B showed only a week *in vitro* cytotoxicity against MM2 cells in the presence of calf serum (50% growth inhibition concentration, IC_{50} = 36 μg/ml), although it was cytotoxic in the absence of the serum (IC_{50} = 1.6 μg/ml) (Fig. 2). This indicates that oenothein B may easily bind to serum components such as proteins, as described for other phenolic compounds.[11] When the tannin is administered *in vivo*, the free fraction is expected to be very small in the animals.

Stimulation of macrophages When oenothein B was intraperitoneally injected into the mice, numerous cells were exuded into the peritoneal cavity with dose-dependency, and the maximum number was found there 4 days after injection of the tannin (Fig. 3). The PEC induced by oenothein B were composed of 60% monocytes, 17% lymphocytes, and 23% polymorphonuclear cells; PEC from normal mice were 62% monocytes, 33% lymphocytes, and less than 5% polymorphonuclear cells. Figure 3 also shows that *in vivo* treatment with oenothein B induced adherent PEC (macrophage-monocytes for the most part), which inhibited ^{3}H-TdR uptake by MM2 cells in a dose-dependent manner with a peak at 4 days after intraperitoneal injection. Macrophages from mice treated with 10 mg/kg oenothein B 4 days earlier not only had strong cytostatic activity (Fig. 3) but also

Table II. Antitumor Effect of Oenothein B on Meth-A Solid-type Tumor in Mice

Agent	Dose (mg/kg)	Tumor weight (g)[a]	% growth inhibition
Oenothein B	10	1.36±0.57[b]	64.9
	3	1.52±0.53[b]	60.7
	1	3.19±0.42	17.6
OK-432	100 (KE/kg)	3.29±0.63	15.0
Control		3.87±0.57	—

a) Mean±SD.
b) Significant difference from the control group, $P<0.05$.

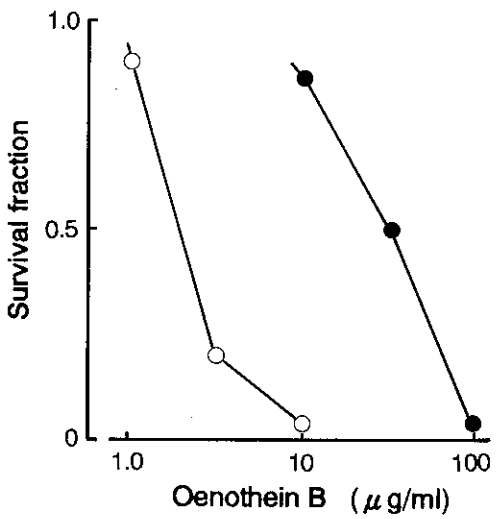

Fig. 2. Direct cytotoxicity of oenothein B on MM2 cells. Cells were treated with graded concentrations of oenothein B in the absence (○) or presence (●) of 10% fetal calf serum for 2 h. The treated cells were incubated for 48 h, and the cell number was counted. Data are the means of triplicate measurements.

Table I. Antitumor Effect of Oenothein B on MM2 Ascites Type Tumor in Mice

Agent	Treatment schedule	Dose (mg/kg)	Survival days[a] (mean±SD)	%ILS	Regressor[b]
Oenothein B	on day −4	10	20	9.3	5[c]
		3	31.8±9.1	73.8	1
		1	19.2±1.9	4.9	0
OK-432	on day −4	100 (KE/kg)	30.0±14.8	63.9	3
Oenothein B	on days 1, 4, 7	10	41.5±0.5	126.8	4[c]
		3	25.7±16.9	40.4	1
		1	26.1±17.0	42.6	0
OK-432	on days 1, 4, 7	100 (KE/kg)	23.0±3.0	25.7	4[c]
Control			18.3±2.4	—	0

a) Excluding 60-day survivors.
b) Number of mice that completely rejected the tumor out of 6 mice in a group at 60 days after the tumor cell inoculation.
c) Significant difference from the control group, $P<0.05$.

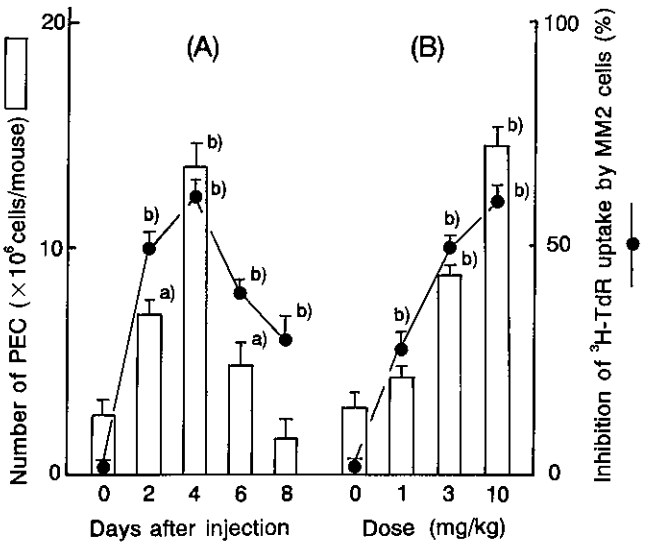

Fig. 3. Changes of number of PEC in mice treated with oenothein B and cytotoxic activity of the adherent PEC on MM2 cells. (A) Changes after intraperitoneal injection of oenothein B (10 mg/kg). (B) Changes 4 days after intraperitoneal injection of the indicated dose of oenothein B. Data are the mean±SE (bar) of triplicate measurements. a) Significant difference from the control, $P<0.05$. b) Significant difference from the control, $P<0.01$.

Table III. Production of IL-1-like Activity by Macrophages of Mice

Treatment of macrophages		IL-1-like activity ($\times 10^3$ cpm)[a]
Non-treated		5.6±1.8
Oenothein B (10 mg/kg, i.p.) before 4 days		20.5±3.7[b]
Oenothein B (*in vitro*)	10 μg/ml	15.9±2.2[b]
	3 μg/ml	13.6±3.7[b]
	1 μg/ml	7.0±1.5
E. coli LPS (*in vitro*)	1 μg/ml	13.2±2.1[b]

a) Mean±SE of triplicate measurements. Mean ^{3}H-TdR uptake on treatment with Con A alone was $26.5\pm0.9\times10^3$ cpm.
b) Significant difference from non-treated control, $P<0.05$.

secreted an IL-1-like activity into the culture medium (Table III).

Then, we examined whether oenothein B stimulates macrophages *in vitro* or not, in comparison with *E. coli* LPS, which is known to induce IL-1 from macrophages.[12] As shown in Tables III and IV, oenothein B induced an IL-1-like activity from mouse macrophages and IL-1β secretion by human peripheral blood macrophages in a concentration-dependent manner.

Table IV. *In vitro* Stimulation by Oenothein B and *E. coli* LPS of IL-1β Release from Human Peripheral Blood Macrophages

Agent		IL-1β (pg/ml[a])
Non-treated		295±66
Oenothein B	30 μg/ml	1440±160[b]
	10 μg/ml	1010±145[b]
	3 μg/ml	730±100[c]
E. coli LPS	10 μg/ml	1230±144[b]
	1 μg/ml	985±171[c]

a) Mean±SE of triplicate measurements.
b) Significant difference from non-treated control, $P<0.01$.
c) Significant difference from non-treated control, $P<0.05$.

DISCUSSION

Various medicinal plants containing tannins have been shown to be effective against cancers and tumors. We have isolated and purified tannins from the plants and found that a group of ellagitannins inhibits the growth of sarcoma S-180 cells in mice, when injected intraperitoneally once, 4 days before the tumor cell inoculation.[2] Among them, oenothein B has a unique macrocyclic structure[7] and is one of the effective tannins. The present study indicates that oenothein B also showed antitumor effect on syngeneic rodent ascites-type (MM2) and solid type (Meth-A) tumors.

The *in vitro* cytotoxicity of this tannin was much less in the presence of calf serum than in the absence of the serum in the culture. Therefore, it is difficult to attribute the antitumor activity of oenothein B to direct cytotoxicity, because the tannin administered to the animal should bind to some entity (e.g., proteins) in the host. Oenothein B induced many PEC after *in vivo* treatment, and the adherent PEC (macrophages) were cytostatic towards MM2 cells and released an LAF (IL-1-like activity). Moreover, this tannin stimulated the release of LAF and IL-1β from macrophages of mice and human, respectively, in a concentration-dependent manner upon *in vitro* treatment. Consequently, oenothein B is thought to play a role as an immunomodulator or immunoregulator in the host. Previously, we suggested that agrimoniin, a non-cyclic ellagitannin, exhibits a host-mediated antitumor activity.[5,6] The action mechanism may be similar to that of oenothein B.

This study indicates that oenothein B is a unique immunomodulatory substance, and its primary mechanism of antitumor activity seems to be direct stimulation of macrophages. This tannin may be available for clinical use, because it stimulated IL-1 release from human macrophages.

(Received July 30, 1992/Accepted September 25, 1992)

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