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SYNTHETIC LIPID A PRODUCES ANTITUMOR EFFECT IN A HAMSTER PANCREATIC CARCINOMA MODEL THROUGH PRODUCTION OF TUMOR NECROSIS FACTOR FROM ACTIVATED MACROPHAGES

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INDEXING WORDS

lipid A derivative; tumor necrosis factor (TNF); macrophage; pancreatic carcinoma

SYNOPSIS

The antitumor effect and biological activities of a newly synthesized lipid A analogue (ONO-4007) were investigated in a hamster pancreatic carcinoma model. Marked and dose-dependent inhibition of tumor growth was achieved by i.p. injection twice a week for 3 weeks of 10, 30 or 50 mg/kg of ONO-4007. Endogenous tumor necrosis factor (TNF) activities induced by ONO-4007 were significantly greater in tumor than in serum, spleen and liver.

TNF production by macrophages stimulated with ONO-4007 after culture was much greater when culture was performed in the presence of hamster pancreatic carcinoma cells (no cell-to-cell contact). It was further found that the cytotoxic activity of TNF secreted by macrophages cultured with cancer cells was inhibited in the presence of anti-TNF neutralizing antibodies. These findings suggest that ONO-4007 displays antitumor effects by stimulating production of

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endogenous TNF in tumor macrophages, possibly through activation by soluble macrophage-stimulating factors in cancer cells.

INTRODUCTION

Lipid A is a hydrophobic component of bacterial lipopolysaccharides (LPS) and has a variety of biological effects including the induction of necrosis and regression of malignancies.[14] Although these actions have received considerable attention, clinical application in cancer therapy has been limited by toxic side effects. A number of lipid A analogues have been synthesized in an attempt to reduce toxicity, some of which have shown beneficial immunopharmacological activities. More recently, a synthetic lipid A subunit, ONO-4007 (sodium 2-deoxy-2- [3S-(9-phenylnonanoyl-oxy)tetradecanoyl] amino-3-*O*-(9-phenylnonanoyl)-D-glucopyranose 4-sulfate) has been shown to have a greatly diminished toxicity less than 1/1000 that of natural *Escherichia coli* LPS, as well as potent antitumor effects against implanted Meth A sarcoma and MH-134 hepatoma in the murine system.[18]

The present study examined the antitumor effect of ONO-4007 in hamster pancreatic carcinoma, which has been intensively characterized as an experimental model analogous to human pancreatic carcinoma.[17] The role of cancer cells in the activation of tumor macrophages was also studied *in vitro*.

MATERIALS AND METHODS

Reagents

ONO-4007 was kindly provided by Ono Pharmaceutical Inc. (Osaka, Japan) and was dissolved in 50% ethanol at 50 mg/ml. Reagents were diluted to the desired concentration with distilled water immediately before each experiment.

Hamster models and pancreatic carcinoma cells

Female Syrian golden hamsters purchased by Japan SLC Co.(Shizuoka, Japan) and aged 6 weeks at the start of the experiments were used. Hamster pancreatic carcinoma was induced by subcutaneous administration of 10 mg/kg of *N*-nitrosobis (2-oxopropyl) amine (BOP) a week for 15 weeks. The tumor in the

pancreas was removed and sliced into cubes of about 1 mm in minimal essential medium (MEM). One tumor cube was inoculated subcutaneously into the rt. axillar region of each hamster and allowed pass through 15 generational cycles. All hamsters were kept at 22±2℃ on a 12 h light-dark cycle, with free access to water and a standard laboratory diet.

To obtain hamster pancreatic carcinoma cells, subcutaneous tumors were minced into small pieces with phosphate-buffered saline (PBS) with 0.25 % trypsin. After filtration through a sterilized nylon mesh, cells were cultured in RPMI 1640 medium with 0.2% L-glutamine, 100 unit/ml penicillin, 100 mg/ml streptomycin and 10% fetal calf serum (FCS) at 37℃ in a humidified 5% CO_2 atmosphere. After culture in a 25 cm^2-flask for 4 weeks, cancer cells predominated over fibroblasts. The cells were detached with 0.25% trypsin and 0.05 M ethylenediamine tetraacetate and were cultured in a 150 cm^2-flask. Cancer cells were cultured through more than twenty-seven generations before use in the present study.

Test of antitumor effect of ONO-4007

Hamster pancreatic carcinomas were transplanted subcutaneous into the right axillar region of each hamster at 6 weeks of age. After 1 week, animals with solid tumors of about 5 mm diameter were selected and given an intraperitoneal injection of 10, 30 or 50 mg/kg of ONO-4007 twice a week (total 6 times). Tumor-bearing hamsters were similarly injected with a solvent as control. Tumor volume (long diameter x short diameter^2 / 2) and body weight were measured every other day.

Extraction for TNF assay

Hamsters in which tumors were about 10 mm in diameter two weeks after transplantation were given an i.p. injection of ONO-4007. One hour after injection, whole blood was collected and centrifuged and the serum used as sample for TNF assay. Briefly, tumors were removed, weighed, minced with scissors and homogenized in PBS containing 10% FCS (0.3 g wet tumor / 1.0 ml). The homogenate was centrifuged at 900 x G for 10 min., and the cloudy supernatant fluid re-centrifuged for 1 h at 100,000 x G to obtain a clear supernatant fluid.[15] The sample solution was stored at -80 ℃ until assay.

TNF activity assay

TNF activity was measured by cytotoxic assay with L929 cells as target. L929 cells at $6x10^4$ cells in 200 μl MEM medium containing 10% FCS and actinomycin D (1 μg/ml) were incubated with diluted samples for 18 h at 37℃ in a humidified 5% CO_2 atmosphere. At the end of the culture period, each well was stained with crystal violet (0.5% in 20% ethanol) for 15 min. The plates were then washed with water and allowed to dry. The stain of crystal violet was extracted using 33% acetic acid and absorbance measured at 570 nm. The value of sample required to destroy 50% of target L929 cells (ED50) was obtained from the dose-response curves using recombinant mouse tumor necrosis factor-α (TNF-α) as standard (Genzyme, Cambridge, MA). TNF activity was expressed as mean units per gram tissue (U/g) of duplicate assay.[11]

Peritoneal macrophages

Resident peritoneal macrophages were prepared by peritoneal lavage from hamsters.[2,5] Hamsters were injected with 15 ml Hank's balanced salt solution (HBSS) in the peritoneal cavity and massaged for one minute on the abdomen. HBSS in hamsters was collected and centrifuged for 10 minutes at 800 x G. Separated cells were washed twice with HBSS and then incubated with RPMI 1640 containing 10% FCS for 2 h at 37℃, 5% CO_2. Macrophages adhered directly to tissue culture dishes and nonadherent cells were washed away.

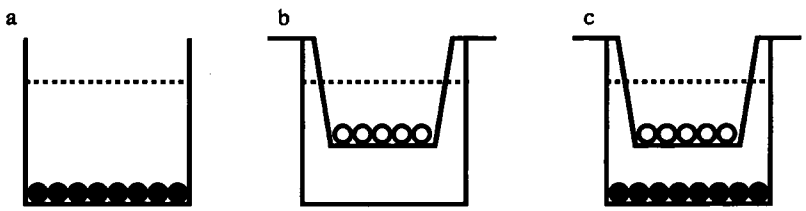

Figure 1. Peritoneal macrophages (PEϕ) and pancreatic carcinoma cells were cultured in differerent conpartments of an insert chamber system (c) with a 0.45 μm pore membrane permeable to medium but not to cells. PEϕ (○) were placed in the upper chamber of the system (b), pancreatic cancer cells (●) in the lower chamber (a).

Culture of macrophages with pancreatic carcinoma cells

Macrophages and tumor cells were cultured in different compartments of a Cell Culture Insert system (Becton Dickinson, Franklin Drive, NJ). The system consisted of upper and lower chambers formed by insertion of a membrane and companion plate. For the experiment, tumor cells were seeded in quadruplicate in the lower chamber (2×10^4/well), and overlaid with 800 μl culture medium. Insert chambers were then placed in the wells and macrophages (1×10^5/200 μl culture medium) cultured in the inserts (Fig. 1).

Cytotoxicity and neutralization assay

Pancreatic carcinoma cells at a density of 3×10^2 cells per well were incubated at 37℃ in 5% CO_2 for 5 days with culture supernatant of macrophages stimulated with ONO-4007 after 48 h culture with cancer cells. As a cytotoxicity neutralization test, supernatant was incubated in addition with murine anti-TNF antibodies (Genzyme). Cytotoxicity was assayed by WST-1 assay.[16)]

Statistics

Statistical analysis was performed using unpaired Student's *t* test.

RESULTS

Antitumor effect of ONO-4007 on pancreatic carcinoma

ONO-4007 showed antitumor effect against pancreatic carcinoma implanted in hamsters (Fig. 2). In animals injected with 10 and 30 mg/kg of ONO-4007, 32 and 38% inhibition of tumor growth were observed 26 days after transplantation, compared with the control group ($p<0.05$). Animals receiving 50 mg/kg of ONO-4007 showed 67% inhibition of tumor growth ($p<0.01$), a significantly greater suppression than other groups.

Body weight showed no decrease and nor was there any significant difference in body weight between groups (Fig.3).

TNF activity in tumors and normal organs

Endogenous tissue TNF activity induced by ONO-4007 was examined intumors and organs. In tumor, serum and spleen, TNF activity peaked 60 min

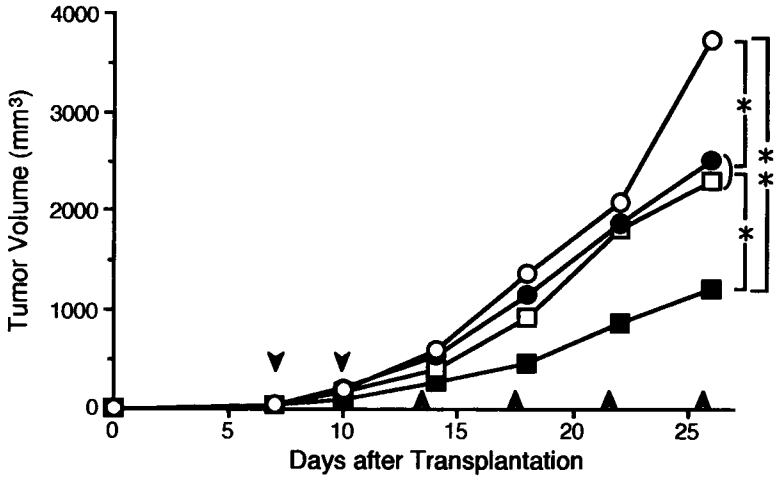

Figure 2. Antitumor effect of ONO-4007 on hamster pancreatic carcinoma. From one week after transplantation of tumor, each hamster was injected twice a week i.p. with 10 (●), 30 (□) or 50 (■) mg/kg of ONO-4007, or solvent as control (○). ▲ : injection of ONO-4007. ＊:P<0.05 ; ＊＊:P<0.01.

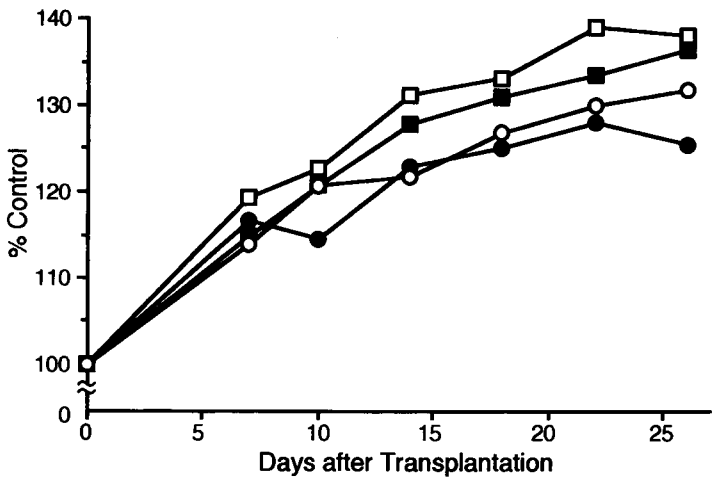

Figure 3. Change of hamster body weight after injection of ONO-4007. Hamsters bearing pancreatic carcinoma were injected twice a week i.p. with 10 (●), 30 (□) or 50 (■) mg/kg of ONO-4007, or solvent as control (○) .

after injection of 50 mg/kg of ONO-4007, while maximum TNF level in the liver was reached 120 min post-injection. Endogenous TNF activity in tumors was greater than in organs at all times, and values higher than maximum values in organs were observed in tumors for up to 120 min after ONO-4007 injection (Table I). TNF activity induced by ONO-4007 in tumors and serum was dose-dependent. The activity induced in tumor tissue was significantly greater than in serum in all groups treated with the reagent ($p<0.05$) (Table II).

Table I. Time course of tissue TNF activity induced by ONO-4007 in each organ.

Organ	TNF activity			
	Time after injection of ONO-4007 (min)			
	30	60	120	180
Tumor (U/g)	367.0 ±167.6	979.0 ±397.4	333.0 ±102.4	42.0±24.3
Serum (U/ml)	8.0 ±7.2	219.0 ±118.8	19.8±11.6	1.2 ±1.0
Liver (U/g)	N.D	6.1±6.4	26.3 ±7.9	1.5 ±0.9
Spleen (U/g)	N.D	38.2 ±28.9	10.0±0.1	3.6±2.1

(N.D.; not detected)

Table II. TNF activity induced by ONO-4007 in tumor and serum.

Organ	TNF activity			
	ONO-4007 (mg/Kg)			
	0	10	30	50
Tumor (U/g)	3.5±3.1	107.6±54.0	207.0±46.6	978.6±355.4
Serum (U/ml)	N.D.	N.D.	4.9±3.5	219.3±118.8

(N.D.; not detected)

TNF activity in macrophages cultured with hamster pancreatic carcinoma cells

TNF activity induced by macrophages in cell culture insert systems is shown in Table III. When stimulated with ONO-4007, TNF induction by macrophages cultured with cancer cells for 24 or 48 h was greater than that by

Table III. TNF activity of macrogages stimulated by ONO-4007, after cultured with pancreatic carcinoma cells using cell culture insert system.

Cultured time with cancer cells (h)	TNF activity (U/ml)			
	ONO-4007 (mg/ml)			
	0.01	0.03	0.1	0.3
macrophage only	2.99±0.56	2.95±0.23	12.35±3.52	7.57±0.39
24	11.02±6.46	9.93±7.41	15.50±4.02	44.54±9.92[a]
48	11.87±3.87[a]	12.90±1.04[a]	94.10±10.34[a]	85.94±27.49[a]

(a:P<0.05 versus macrophage only)

macrophages cultured without tumor cells. TNF activity in macrophages cultured for 48 h was higher than in those cultured for 24 h. In addition, TNF activity was not induced in macrophages cultured without tumor cells, even when stimulated with 0.3 mg/ml of reagent. TNF activity induced in pancreatic cancer cells alone was not detected (data not shown).

Cytotoxicity of macrophages against cancer cells

Culture supernatant of macrophages stimulated with 0.3 mg/ml of ONO-4007 after culture with hamster pancreatic carcinoma cells showed high cytotoxicity against cancer cells *in vitro*. This cytotoxic activity correlated with TNF activity, and was partly inhibited by anti-TNF neutralizing antibodies when 0.1 or 0.3 mg/ml of ONO-4007 was used (Fig. 4).

DISCUSSION

LPS and its active component, lipid A, have been recognized as having a variety of biological effects including anti-tumor effect.[14] The greatest barrier to clinical use as an anti-tumor drug is serious toxicity in normal tissue. A new synthetic derivative of lipid A, ONO-4007, has been developed to dissociate the effectiveness from the toxicity. In the present study, significant and dose-dependent inhibition of tumor growth without harmful side effects was observed following i.p. injection of ONO-4007 in hamsters bearing pancreatic carcinoma. TNF-α has been reported to act against several kinds of cancer cell *in*

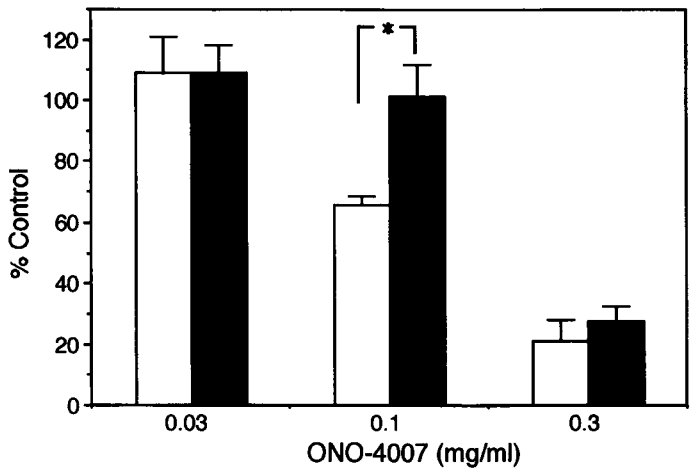

Figure 4. Cytotoxicity and neutralization test of supernatant of activated macrophages. Pancreatic carcinoma cells (3 x 102 / well in 96-well microplate) were cultured for 5days with supernatant of macrophages stimulated with the indicated dose of ONO-4007 (□). In cytotoxicity neutralization test, culture medium was pre-incubated in addition with 100-times diluted murine anti-TNF antibodies (■). Cancer cells were cultured with supernatant of macrophages without stimulation of the agent as control. Cytotoxicity was assayed by WST-1 assay. * : $P<0.05$

vitro [12,13] and has been applied to some extent to clinical use in cancer patient, [1,3] but with little effect on account of the difficulty of elevating TNF level in tumor tissue. Tables II and III, endogenous TNF production in tumor tissue was greater than in blood or normal tissue in the major important organs, and was dependent on dose of ONO-4007. These findings suggest that ONO-4007 may inhibit the growth of hamster pancreatic carcinoma by inducing endogenous TNF in tumor tissue. However, the antitumor effect of macrophages induced by ONO-4007 may not be due to TNF alone. Macrophages play an important role in host defense against tumors through their ability to synthesize and secrete a variety of immunoregulatory and inflammatory cytokines: interleukin (IL)-1, IL-6, and TNF. Recent reports have demonstrated that activated macrophages

revitalize T cell-mediated immunological activity and that cytotoxic T cells may actually contribute to antitumor effect.[6,7] Activated macrophages produce nitric oxide (NO), which inhibits cancer cells via an action dependent on L-arginine.[4,9] Hattori *et al.* reported that ONO-4007 induced NO synthase *in vitro* and *in vivo*.[8] NO as well as TNF may thus play an important role in the antitumor effect of ONO-4007.

In mouse MM-46 tumor, activation by ONO-4007 of tumor-infiltrating macrophages and the resultant substantial TNF production has been demonstrated to exert antitumor effect.[18] Macrophages in the normal state have little tumoricidal activity, but in the activated state may exhibit cytocidal action on cancer cells, that is, they may require two kinds of signal to be activated, a priming signal and a triggering signal.[10] It is not clear, however, by what mechanism macrophages in tumor tissue are activated and whether TNF production induced by ONO-4007 actually contributes to its antitumor effect. TNF induction by 0.1 or 0.3 mg/ml of ONO-4007 was maredly higher in macrophages previously cultured with cancer cells for 24 or 48 h *in vitro*; macrophages cultured without cancer cells were not stimulated even by 0.3 mg/ml of agent. The culture medium of macrophages cultured with cancer cells had a cytotoxic activity against hamster pancreatic cancer cells which was dependent on TNF activity in the medium, and was neutralized addition of anti-TNF antibodies. These findings suggest that activation of macrophages requires the presence not only of ONO-4007 but also of pancreatic cancer cells, and that macrophages may provide antitumor effect via TNF production and release. Pancreatic cancer cells could thus be considered to act as macrophage priming agents. This effect was obtained without cell-to-cell contact with tumor cells, which means that soluble mediators such as macrophage- activating cytokines - IL-1, interferon-γ or macrophage colony-stimulating factor- are involved.

In conclusion, it was shown that ONO-4007 displayed antitumor effect stimulating production of endogenous TNF in macrophages in a hamster pancreatic carcinoma model. It was also indicated that macrophages in the tumors may be activated by soluble macrophage-stimulating factors secreted by cancer cells. Future study is needed of activation of T cells and NO synthase by ONO-4007 in a hamster models. It will also be necessary to identify the cancer-derived soluble factors acting as priming signals for macrophages.

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