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A new synthetic lipid A analog, ONO-4007, stimulates the production of tumor necrosis factor-α in tumor tissues, resulting in the rejection of transplanted rat hepatoma cells

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ONO-4007 is a new synthetic lipid A derivative with low endotoxic activities. We have examined the therapeutic effects of ONO-4007 on rat hepatocellular carcinoma KDH-8 cells, rat fibrosarcoma KMT-17 cells and rat mammary adenocarcinoma SST-2 cells *in vivo*. Multiple systemic i.v. administration of ONO-4007 was performed on days 7, 14 and 21 after tumor implantation of KDH-8 and SST-2 cells, and on days 5, 10 and 15 after tumor implantation of KMT-17 cells. ONO-4007 showed significant therapeutic effects on KDH-8 cells; by the administration of ONO-4007 (2.5 mg/kg) 70% of rats were cured and by the administration of ONO-4007 (5 mg/kg) 50% of rats were cured. Furthermore, the ONO-4007 treatment prolonged the mean survival time of KDH-8-bearing rats. However, ONO-4007 had no effect on KMT-17 and SST-2 cells, and it had no direct effect on the growth of KDH-8 cells *in vivo*. Albeit the stimulation with ONO-4007 induced mRNA expressions of interleukin (IL)-1α, IL-6 and tumor necrosis factor (TNF)-α, those of IL-2, IL-4, IL-10 and interferon (IFN)-γ were not induced. Using a bioassay, we found that the production of TNF-α in the tumor tissues was induced by ONO-4007 in a dose-dependent manner. KDH-8 cells were sensitive to human natural TNF-α *in vitro*. However, KMT-17 and SST-2 cells were resistant against TNF-α *in vitro*. These results suggest that ONO-4007 is therapeutically useful for the treatment of TNF-α-sensitive tumors.

Key words: Hepatocellular carcinoma, lipid A, lipopolysaccharide, tumor necrosis factor-α.

Introduction

Lipopolysaccharide (LPS) in a Gram-negative bacterial envelope is a potent stimulator for many early events in macrophage activation, such as the production and secretion of interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α.[1,2] Many investigators showed that LPS also had potent antitumor activities.[3,4] However, since LPS causes severe side effects, it is not used clinically yet. Lipid A, a hydrophobic component of bacterial LPS, is known to be a biologically active site of LPS. Many synthetic lipid A derivatives have been developed to be less harmful to hosts, but the expected new derivatives did not satisfy both potent immunopharmacological activities and low toxicity.[5–7]

Tumor necrosis factor (TNF)-α is a regulatory protein secreted mainly by activated macrophages and is selectively cytotoxic to tumorigenic or transformed cells, but not against normal cells.[8] Lipid A is thought to be a potent factor to induce endogenous TNF-α in a tumor bearer. Therefore a low toxic lipid A is expected to be an immunomodulator inducing TNF-α production *in vivo*.

A novel lipid A derivative, ONO-4007, was developed, which represents low toxicity less than 1/1000 that of natural *Escherichia coli* LPS.[9] *In vivo* treatment i.v. administration of ONO-4007 brought about therapeutic effects on the rats inoculated with leukemic cells without severe side effects.[10] Here, we describe the antitumor effects of ONO-4007 on rat hepatocellular carcinoma KDH-8 cells, rat fibrosarcoma KMT-17 cells and rat mammary adenocarcinoma SST-2 cells *in vivo* and *in vitro*.

Materials and methods

Animals

Female Wister King Aptekman (WKAH) rats 8–12 weeks old were supplied by the Experimental Ani-

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mal Institute of the Hokkaido University School of Medicine, Sapporo, Japan. Female spontaneously hypertensive rats (SHR) 8–12 weeks old were supplied by the Nippon Rat Co. (Urawa, Japan). The animals were kept in a room with controlled temperature, humidity and 12 h light/dark cycle. Food and water were supplied *ad libitum*.

Cell lines

KDH-8 is a rat transplantable hepatocellular carcinoma induced by 3'-methyl-4-dimethylaminoazo-benzene in a WKAH rat and maintained *in vivo* by i.p. passage every 5 days.[11] KMT-17 is a transplantable rat fibrosarcoma line induced by 3-methylcholanthrene in a WKAH rat and maintained *in vivo* by i.p. passage every 3 days. KMT-17/A3 is a subclone of the *in vitro* KMT-17 parental line and exhibits properties similar to those of the parent *in vivo* line. KMT-17/A3 cells were maintained in a continuous *in vitro* culture in RPMI 1640 medium supplemented with 10% FBS.[12] SST-2 is a transplantable adenocarcinoma line that originated from a spontaneous mammary adenocarcinoma in a SHR. SST-2 cells were maintained in a continuous *in vitro* culture in Eagle's minimum essential medium supplemented with 10% FBS.[13]

Reagents

ONO-4007 (sodium2-deoxy-2-[3s-(9-phenylnonayloxy)tetradecanoyl]-amino-3-*O*-(9-phenylnona-nonyl)-D-glucopyranose-4-sulfate) was kindly provided by ONO Pharmaceutical (Osaka, Japan). Figure 1 shows its chemical structure. For *in vivo* use ONO-4007 was dissolved in 50% ethanol at 50 mg/ml, diluted with distilled water to appropriate concentrations. For *in vitro* use ONO-4007 was dissolved in dimethyl sulfoxide (DMSO) at 50 mg/ml, diluted with RPMI 1640 medium supplemented with 10% FBS. Human natural TNF-α was a gift from the Department of Neurosurgery, Hokkaido University School of Medicine.

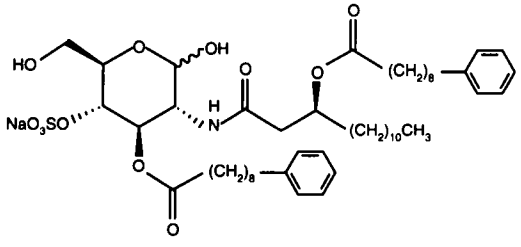

Figure 1. Chemical structure of ONO-4007.

In vivo antitumor effects

On day 0, 1×10^5 KDH-8, KMT-17 and SST-2 cells were transplanted s.c. in WKAH rats or SHR rats. On days 7, 14 and 21, different doses of ONO-4007 (0, 1, 2.5 and 5 mg/kg) or saline were administered i.v. to the rats. Tumor size was measured every other day. Tumor-bearing rats were observed up to 90 days after tumor transplantation when all survivors had no tumor.

Preparation of tissue homogenate

The rats were killed 90 min after the treatment on day 21. The tumor tissues and spleens were resected and homogenized in RPMI 1640 medium supplemented with 10% FBS, 1 ml per 27 mg portion. After centrifugation at 20 000 *g* for 60 min, they were passed through 0.45 μm pore size filters and used for TNF bioassay. The sera were pooled and stored at −20°C until use.

Bioassay for TNF-α

The TNF-α assay employed in this experiment was the 1-day assay using a L-929, TNF-α-sensitive murine fibroblast cell line, in the presence of 0.2 μg/ml actinomycin D.[14] Test samples were obtained from homogenate of the tumor tissues after administration of ONO-4007.

Reverse transcription-polymerase chain reaction (RT-PCR)

The rats were killed 90 min after the treatment on day 21. Total RNAs were extracted from the tumor tissues treated with or without ONO-4007 by the guanidine thiocyanate–phenol–chloroform method. Five micrograms of each RNA sample underwent cDNA synthesis in 50 μl of reaction mixture containing 75 mM KCl, 50 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM dithiothreiotol, 0.5 mM per each dNTP, 2 μg/ml random primer

and 1000 U MMLV reverse transcriptase (Gibco/BRL, Gaithersburg, MD) by incubation at 37°C for 1 h. PCR amplification of cDNA (5 μl) was performed in 50 μl of containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 2.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each dNTP, 10 mM per each specific primer, and 1 U Taq polymerase (Promega, Madison, WI).

The following primer pairs were used. IL-1α:[15] 5′ primer, GTGGTGGTGTCAGCAACATC; 3′ primer, CCTTCAGCAACACAGGCTTG. IL-2:[16] 5′ primer, CTCCTGAGAGGGATCGATAA; 3′ primer, ATGGCTATCCATCTCCTCAG. IL-4:[17] 5′ primer, TAGGACATGGAAGTGC; 3′ primer, ATGGGTCTCAGCCCCCAC. IL-6:[18] 5′ primer, CACCCACAACAGACCAGTAT; 3′ primer, GAGTAGACCTCATAGTGACC. IL-10:[19] 5′ primer, CATGCCTGGCTCAGCACTGC; 3′ primer, GGGAACTGAGGTATCAGAGG. IFN-γ:[20] 5′ primer, ATCTGGAGGAACTGGCAAAAGGACG; 3′ primer, CCTTAGGCTAGATTCTGGTGACAGC. TNF-α:[21] 5′ primer, CAAGGAGGAGAAGTTCCCAA; 3′ primer, CGGACTCCGTGATGTCTAAG. GAPDH:[22] 5′ primer, ACCACCATGGAGAAGGCTGC; 3′ primer, CTCAGTGTAGCCCAGGATGC. In principle, the primer sequence were chosen from separate DNA exons of the gene. Expected sizes of amplified DNA fragments were 412, 305, 478, 509, 682, 288, 500 and 500 bp for IL-1α, IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α and GAPDH, respectively. The reactions were run for 25, 30 and 35 cycles using a thermal cycler as follows: 1 min at 94°C, 1 min at 60°C and 2 min at 72°C. Nine microliters of each PCR sample was mixed with 1 μl of the sample buffer, electrophoresed through 1% agarose gel and stained with ethidium bromide.

Cytotoxicity assays

Cytotoxic assays were performed by a colorimetric MTT assay. Then, 1×10^4 cells were plated in 100 μl of RPMI 1640 medium supplemented with 10% FBS in 96-well flat-bottomed microplates and treated with various doses of ONO-4007 or natural human TNF-α. The cultures were preincubated for 48 h and, for the final 6 h, incubated with 500 μg/ml of MTT [1-(4,5-dimethylthiazol-2,5-diphenyl) tetrazolium bromide] (Sigma, St Louis, MO). After 100 μl of isopropanol containing 0.04 N HCl was added to each well and mixed throroughly to dissolve the dark blue crystals, the plates were read on a MTP-100 microplate reader (Corona Electric, Katsuta, Japan) at a test wavelength of 570 nm with a reference wavelength of 610 nm.

Statistical analysis

Statistical determinations, where applied, were calculated by the Student's *t*-test, χ^2-test and generalized Wilcoxon test.

Results

Therapeutic effects of ONO-4007 on hepatocellular carcinoma KDH-8 in WKAH rats

Figure 2 shows the individual growth curves of KDH-8 in WKAH rats treated with different doses of ONO-4007 as described in Materials and methods. In untreated rats the average diameter of the tumors reached more than 40 mm. Although no untreated rats survived more than 45 days, some ONO-4007-treated rats survived up to 90 days. Figure 3 shows the survival curves of WKAH rats bearing KDH-8 after the treatment. Although no untreated rat survived more than 90 days, 10% of the rats treated with 1 mg/kg of ONO-4007 ($p < 0.001$), 70% of the rats treated with 2.5 mg/kg of ONO-4007 ($p < 0.001$) and 50% of the rats treated with 5 mg/kg of ONO-4007 ($p < 0.01$) survived. Furthermore the mean survival time of the rats treated with 1 and 2.5 mg/kg of ONO-4007 was longer than that of untreated rats (Figure 3). Severe side effects were observed in only one rat treated with 5 mg/kg of ONO-4007.

Therapeutic effects of ONO-4007 on fibrosarcoma KMT-17 in WKAH rats and mammary adenocarcinoma SST-2 in SHR rats

Tables 1 and 2 show lethal growth incidences of KMT-17 and SST-2 cells in the rats untreated or treated with different doses of ONO-4007. ONO-4007 had no therapeutic effects on KMT-17 and SST-2-bearing rats.

In vitro direct cytotoxic effects of ONO-4007 on KDH-8 cells

Table 3 shows direct cytotoxicity of ONO-4007 on KDH-8 cells *in vitro* by the MTT assay. ONO-4007 could not inhibit *in vitro* growth of KDH-8 cells up to the concentration of 10 μg/ml. From these results, it was shown that the therapeutic effects of

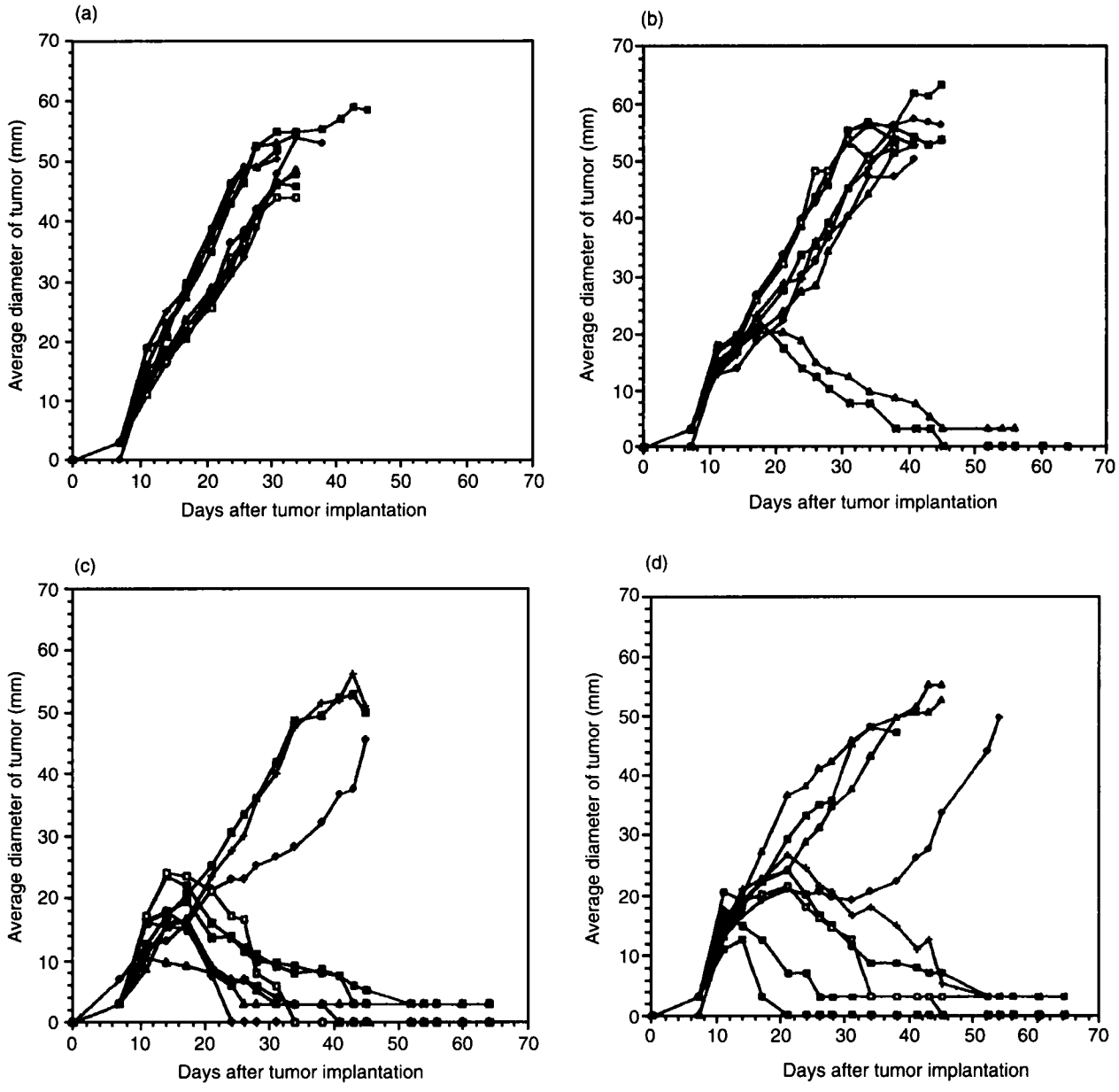

Figure 2. Individual growth curves of rat hepatoma KDH-8 in WKAH rats treated with various doses of ONO-4007 *in vivo*. (A) Saline-treated rats (saline i.v.; $n = 10$). (B) ONO-4007-treated rats (1 mg/kg i.v.; $n = 10$). (C) ONO-4007-treated rats (2.5 mg/kg i.v.; $n = 10$). (D) ONO-4007-treated rats (5 mg/kg i.v.; $n = 10$). KDH-8 cells (1×10^5) were s.c. implanted in WKAH rats on day 0. On days 7, 14 and 21 the rats were treated with ONO-4007 (1, 2.5 and 5 mg/kg i.v.). Tumor size was measured every other day. Tumour-bearing rats were observed till their death.

ONO-4007 on KDH-8 tumor-bearing rats were not due to its direct cytotoxicity against tumor cells.

The effects of ONO-4007 on mRNA expression of cytokines in tumor tissues

Figure 4 shows cytokine mRNA expression in tumor tissues of rats treated with or without ONO-4007 after 30 cycles of amplification by RT-PCR. Expected sizes of 412 bp (IL-1α), 305 bp (IL-2), 478 bp (I-4), 509 bp (IL-6), 682 bp (IL-10), 288 bp (IFN-γ), 500 bp (TNF-α) and 500 bp (GAPDH) were amplified. The mRNA expression of IL-2 and IL-4 was not detectable. Tumor tissues from untreated rats showed only faint bands of IL-1α, IL-6 and TNF-α. In tumor tissues of rats treated with ONO-4007, clear bands of IL-1α, IL-6 and TNF-α were observed, while no change was

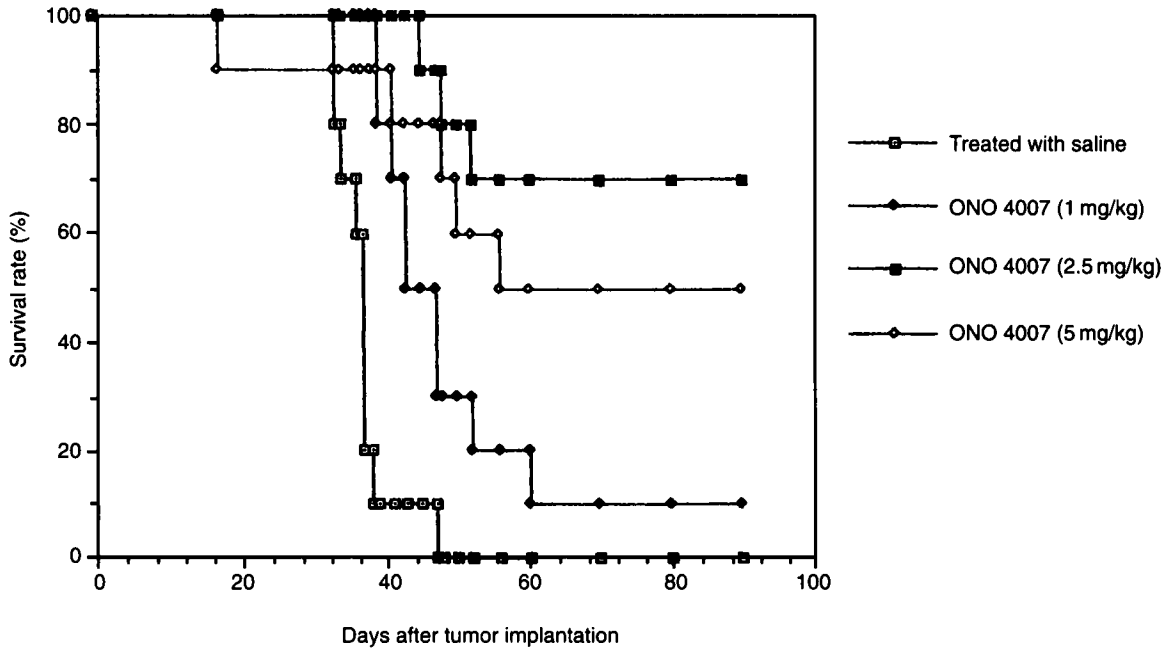

Figure 3. Survival curves of KDH-8 tumor-bearing rats treated with ONO-4007. KDH-8 cells (1×10^5) were s.c. implanted in WKAH rats on day 0. On days 7, 14 and 21 the rats were treated with ONO-4007 (1, 2.5 and 5 mg/kg i.v.; $n = 10$) or saline. ONO-4007 treatment (1, 2.5 and 5 mg/kg i.v.) prolonged the survival time of KDH-8-bearing rats ($p < 0.001$, $p < 0.001$, $p < 0.01$, respectively).

Table 1. The effects of ONO-4007 on fibrosarcoma KMT-17 cells[a] in WKAH rats

Treated with ONO-4007 (mg/kg)[b]	Died/treated (%)	Mean survival time (days ±SD)
No	5/5 (100)	15.6 ± 2.1
Yes (1)	5/5 (100)	16.5 ± 4.6
Yes (2.5)	4/5 (80)	20.5 ± 8.5
Yes (5)	4.5 (80)	14.2 ± 1.5

[a]KMT-17 cells (1×10^5) were transplanted s.c. in WKAH rats on day 0.
[b]ONO-4007 (1, 2.5 and 5mg/kg) or saline was administered i.v. to rats on days 5, 10 and 15.

Table 2. The effects of ONO-4007 on rat mammary adenocarcinomas SST-2[a] cells in SHR rats

Treated with ONO-4007 (mg/kg)[b]	Died/treated (%)	Mean survival time (days ±SD)
No	5/5 (100)	40.2 ± 7.5
Yes (1)	5/5 (100)	40.8 ± 7.5
Yes (2.5)	5/5 (100)	35.4 ± 5.8
Yes (5)	5/5 (100)	40.2 ± 8.6

[a]SST-2 cells (1×10^5) were transplanted s.c. in SHR rats on day 0.
[b]ONO-4007 (1, 2.5 and 5 mg/kg) or saline was administered i.v. to rats on days 7, 14 and 21.

Table 3. The effects of ONO-4007 on *in vitro* growth of hepatocellular carcinoma KDH-8 cells

Concentration of ONO-4007 (μg/ml)	OD (% inhibition)
10	0.80 ± 0.01 (−2.6)
1	0.85 ± 0.04 (−9.0)
0.1	0.85 ± 0.02 (−9.0)
0.01	0.88 ± 0.01 (−12.8)
0.001	0.87 ± 0.02 (−11.5)
0	0.78 ± 0.01

These data obtained by a colorimetric MTT assay. KDH-8 cells (1×10^4) were plated in 100 μl of medium in 96-well flat-bottomed microplates and treated with ONO-4007. The cultures were preincubated for 48 h and, for the final 6 h, incubated with 500 μg/ml of MTT. After 100 μl of isopropanol containing 0.04 N HCl was added to each well and mixed, the plates were read on a MTP-100 microplate reader at a test wavelength of 570 nm with a reference wavelength of 610 nm.

observed in mRNA expression of IL-2, IL-4, IL-10 and IFN-γ in tumor tissues of rats treated with ONO-4007. GAPDH mRNA expression was observed to the same extent in all tumor tissues. These results indicate that ONO-4007 stimulated mRNA expression of IL-1α, IL-6 and TNF-α in tumor tissues of the rats.

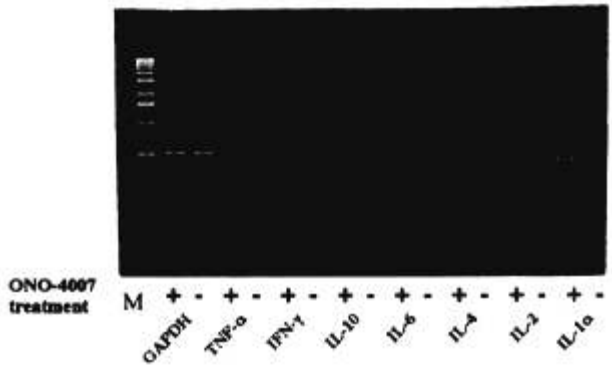

Figure 4. The effects of ONO-4007 on mRNA expression of IL-1, IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α in KDH-8 tumor tissues. The figure shows the bands stained with ethidium bromide. Total RNAs extracted from the KDH-8 tumor tissues of rats treated with ONO-4007 (2.5 mg/kg i.v.) or saline were analyzed by RT-PCR. Expected sizes of DNA fragments for IL-1α, IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α and GAPDH were amplified.

The effects of ONO-4007 on TNF-α production in tumor tissues

The results of RT-PCR showed that ONO-4007 can enhance mRNA expression of monokine, IL-1α, IL-6 and TNF-α in tumor tissues of the rats. TNF-α production was monitored because it is a tumoricidal monokine produced by monocytes/macrophages. Figure 5 shows that ONO-4007 induced TNF-α production in tumor tissues 90 min after the treatment in a dose-dependent manner. The tumor tissues of the rats treated with ONO-4007 (5 mg/kg) contained more than 500 unit/ml of TNF-α.

The effects of ONO-4007 on TNF production in spleens and sera

Figure 6 shows that ONO-4007 (2.5 mg/kg, i.v.) did not induce TNF-α production in spleens and sera 90 min after the treatment.

In vitro cytotoxic effects of TNF-α on KDH-8 cells

The results of the TNF-α bioassay showed that ONO-4007 induced TNF-α production in tumor tissues. To elucidate the relation between TNF-α production in tumor tissues and therapeutic effects of ONO-4007, we examined the effects of TNF-α on *in vitro* growth of KDH-8 cells. Table 4 shows that KDH-8

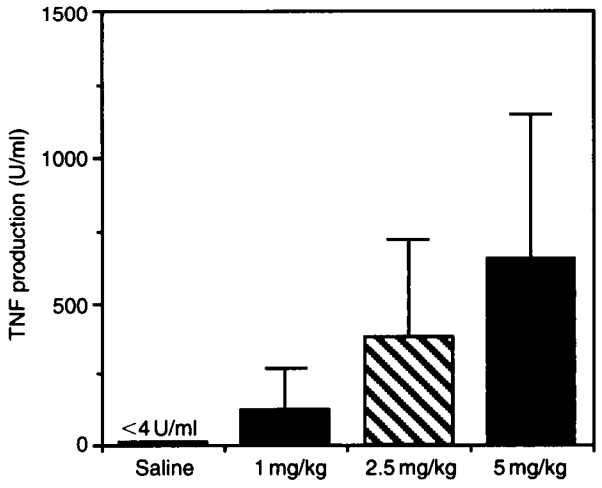

Figure 5. The effects of ONO-4007 on TNF-α production in KDH-8 tumor tissues. KDH-8 cells (1×10^5) were s.c. implanted in WKAH rats on day 0. On days 7, 14 and 21 the rats were treated with ONO-4007 (1, 2.5 and 5 mg/kg i.v.; $n = 3$) or saline. They were killed 90 min after the treatment on day 21. The tumor tissues were resected and homogenized in RPMI 1640 medium supplemented with 10% FBS, 1 ml per 27 mg portion. After centrifugation at 20 000 *g* for 60 min, they were passed through 0.45 μm pore size filters and used for the TNF-α bioassay.

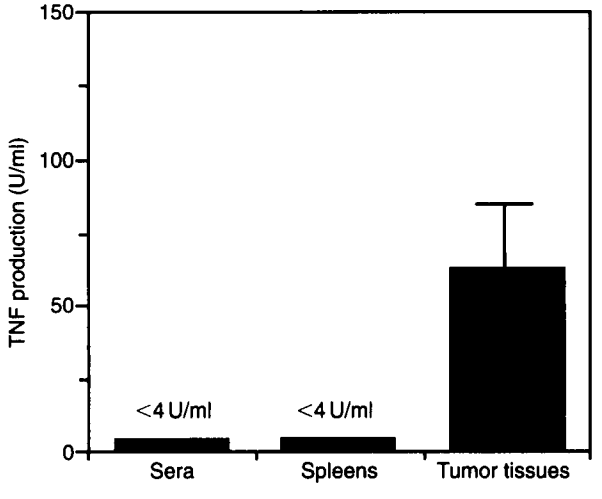

Figure 6. The effects of ONO-4007 on TNF-α production in KDH-8 tumor tissues, spleens and sera. KDH-8 cells (1×10^5) were s.c. implanted in WKAH rats on day 0. On days 7, 14 and 21 the rats were treated with ONO-4007 (2.5 mg/kg i.v.; $n = 3$). They were killed 90 min after the treatment on day 21. The tumor tissues and spleens were resected and homogenized in RPMI 1640 medium supplemented with 10% FBS, 1 ml per 27 mg portion. After centrifugation at 20 000 *g* for 60 min, they were passed through 0.45 μm pore size filters and used for the TNF-α bioassay. The sera were pooled and stored at −20°C until use.

Table 4. The effects of natural human TNF-α on *in vitro* growth of KDH-8, KMT-17/A-3 and SST-2 cells

	Concentration of TNF-α (U/ml)	OD (% inhibition)
KDH-8	20.0	0.58 ± 0.01^a (16.0)
	10.0	0.62 ± 0.03^a (10.1)
	5.0	0.60 ± 0.02^a (13.0)
	2.5	0.63 ± 0.01^a (8.7)
	1.25	0.63 ± 0.02^a (8.7)
	0	0.69 ± 0.01
KMT-17/A-3	20.0	0.60 ± 0.01 (−11.1)
	10.0	0.60 ± 0.04 (−11.1)
	5.0	0.60 ± 0.03 (−11.1)
	2.5	0.56 ± 0.04 (−3.7)
	1.25	0.59 ± 0.02 (−9.3)
	0	0.54 ± 0.03
SST-2	20.0	0.86 ± 0.01 (2.3)
	10.0	0.87 ± 0.03 (1.1)
	5.0	0.83 ± 0.03 (4.5)
	2.5	0.83 ± 0.05 (4.5)
	1.25	0.85 ± 0.04 (2.3)
	0	0.88 ± 0.02

These data were obtained by a colorimetric MTT assay. Tumor cells were plated in 100 μl of medium in 96-well flat-bottomed microplates and treated with human natural TNF. The cultures were preincubated for 48 h and, for the final 6 h, incubated with 500 μg/ml of MTT. After 100 μl of isopropanol containing 0.04 N HCl was added to each well and mixed, the plates were read on a MTP-100 microplate reader at a test wavelength of 570 nm with a reference wavelength of 610 nm.
[a] $p < 0.01$ versus control as analyzed by Student's *t*-test.

cells were sensitive to TNF-α. TNF-α inhibited KDH-8 cell growth ($p < 0.01$). However, KMT-17/A3 and SST-2 cells were resistant against TNF-α. These results suggest that ONO-4007 exerts therapeutic effects only on the rats bearing TNF-α-sensitive tumor cells.

Discussion

The results of the present study indicate that a new synthetic lipid A derivative with low endotoxic activities, ONO-4007, had significant potent therapeutic effects for rat hepatocelluar carcinoma KDH-8-bearing rats, but was not effective for rat fibrosarcoma KMT-17- and mammary adenocarcinoma SST-2-bearing rats. Severe side effects were observed only in one rat treated with 5 mg/kg of ONO-4007. ONO-4007 did not inhibit *in vitro* growth of KDH-8 cells by doses up to 10 μg/ml. The tumor tissues of the rats treated with ONO-4007 produced TNF in a dose-dependent manner, whereas we could not detect TNF-α production in tumor tissues of the untreated tumor-bearing rats. However, we could not detect TNF-α activity in sera and spleen tissues of the rats 90 min after the administration of ONO-4007. Recombinant TNF-α inhibited the growth of KDH-8 cells *in vitro*, but did not inhibit the growth of KMT-17/A3 cells and SST-2 cells. These results suggest that ONO-4007 can be used *in vivo* as a biological response modifier (BRM) for TNF-α-sensitive tumor bearers.

Although we detected much production of TNF-α in tumor tissues, we did not detect it in spleens and sera in the tumor-bearing rats 90 min after the treatment with ONO-4007. Since TNF-α is toxic to a host, higher serum TNF-α is not advantageous. The fact that only one rat suffered from the severe side effects of ONO-4007 shows the advantage of lower serum TNF-α in ONO-4007-treated rats than in the treated rats. Production of TNF-α only in tumor tissues is very important for the inhibition of tumor growth by TNF-α. Gatanaga *et al.*[23] reported that intratumoral injection of a low dose of recombinant TNF-α shows higher antitumour effects than systemic administration of a high dose of it. This suggests that the local accumulation of stable amounts of TNF-α is important for TNF-α biotherapy. Koshita *et al.*[24] recently studied the antitumor effects of human TNF-α gene-transfected murine fiblosarcoma Meth A on parent Meth A-bearing mice. Their results showed that the inhibition of tumor growth required locally secreted TNF-α but not a high serum TNF-α. Based on these findings, the intratumoral TNF-α seems to play a major role in the antitumor mechanisms of ONO-4007. We could not elucidate why TNF-α was produced only in tumor tissues by treatment with ONO-4007. Many monocytes are resident in spleens; therefore, the difference in numbers of monocytes/macrophages in the spleens and the tumor tissues may not explain the difference of TNF-α production in spleens and tumor tissues. The particular environment in the tumor tissue may induce increased TNF-α production from monocytes/macrophages by ONO-4007 treatment.

We showed that TNF-α induced in tumor tissues by ONO-4007 was important in biotherapy for TNF-α-sensitive tumor bearers. However, we are not able to account for whether these therapeutic effects of ONO-4007 are due to the direct inhibition of TNF-α against tumor cells. Even though 20 unit/ml of TNF-α inhibited only 16.0% of KDH-8 cell growth, TNF-α might be a trigger for the cytokine network cascade and nitric oxide production might contribute partly to the effects of ONO-4007. It is necessary to determine whether TNF-α production in tumor

tissues is an important event for the antitumor effects of ONO-4007 by blocking experiments using anti-TNF-α antibody *in vivo*.

In conclusion, our present study has demonstrated that a new synthetic lipid A analog, ONO-4007, had therapeutic effects for TNF-sensitive tumors. Although the mechanism of rejection of TNF-α-sensitive tumors by treatment with ONO-4007 is yet to be elucidated in detail, our study suggests that ONO-4007 is therapeutically useful for the treatment of TNF-α-sensitive tumors.

Conclusion

A new lipid A analog, ONO-4007, showed significant therapeutic effects on rat hepatoma KDH-8, but had no effect on KMT-17 and SST-2. Production of TNF-α in the tumor tissues was induced by ONO-4007. KDH-8 cells were sensitive to human natural TNF-α *in vitro*, but KMT-17 and SST-2 cells were resistant. ONO-4007 is expected as a new BRM for the treatment of TNF-α-sensitive tumors.

Acknowledgments

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