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Short report

Therapeutic effects of a new synthetic lipid A analog, ONO-4007, on rat hepatoma KDH-8 depend on tumor necrosis factor-sensitivity of the tumor cells

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ONO-4007 is a new synthetic lipid A derivative with low endotoxic activities. ONO-4007 was effective against KDH-8, a tumor necrosis factor (TNF)-sensitive rat hepatoma cell line, but neither effective against KMT-17, a TNF-resistant rat fibrosarcoma cell line, nor SST-2, a TNF-resistant rat mammary adenocarcinoma cell line. We have established two sublines from KDH-8 to further examine the therapeutic mechanisms of ONO-4007 *in vivo*: TNF-sensitive KDH-8/YK and TNF-resistant cKDH-8/11. The two sublines equally proliferated *in vitro*. Multiple systemic i.v. administration of ONO-4007 was performed on days 7, 14 and 21 after tumor implantation. Although treatment with ONO-4007 had no effect on the growth of cKDH-8/11 in WKAH rats *in vivo*, 60% of KDH-8/YK-bearing rats treated with ONO-4007 survived. The administration of ONO-4007 brought about significant therapeutic effects on KDH-8/YK-bearing rats but not on cKDH-8/11-bearing rats. These results suggest that ONO-4007 is therapeutically useful for the treatment of TNF-α-sensitive tumors.

Key words: Hepatoma, lipid A, lipopolysaccharide, tumor necrosis factor.

Introduction

Lipopolysaccharide (LPS) is a potent stimulator to activate macrophages[1,2] and has potent antitumor activities.[3,4] Lipid A, a hydrophobic component of bacterial LPS, is known to be a biologically active site of LPS and to have severe side effects. In contrast, a newly developed lipid A analog, ONO-4007, has low toxicity, less than 1/1000 that of natural *Escherichia coli* LPS.[5] *In vivo* treatment with i.v. administration of ONO-4007 brought about therapeutic effects on the rats inoculated with syngeneic hepatoma KDH-8 cells without severe side effects. However, ONO-4007 was not effective against fibrosarcoma KMT-17 and mammary adenocarcinoma SST-2. It was found that ONO-4007 induced the production of tumor necrosis factor (TNF)-α in KDH-8 tumor tissues in a dose-dependent manner and that KDH-8 cells were sensitive to TNF-α *in vitro*, whereas 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.[6]

In this study, we have established a TNF-sensitive line, KDH-8/YK, and a TNF-resistant line, cKDH-8/11, from KDH-8, and examined the antitumor effects of ONO-4007 on the these lines *in vitro*.

Materials and methods

Animals

Female Wister King Aptekman/Hok (WKAH) rats aged 8–12 weeks were supplied by the Experimental Animal Institute, Hokkaido University School of Medicine, Sapporo, Japan. The animals were kept in a room with controlled temperature, humidity and a 12 h light/dark cycle. Food and water were supplied *ad libitum*.

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Cell lines

KDH-8 is a rat transplantable hepatocellular carcinoma induced by 3'-methyl-4-dimethylaminoazo-benzene in a WKAH rat and has been maintained *in vivo* by i.p. passage every 5 days.[6] KDH-8/YK is a cell line isolated from the primary culture of KDH-8 tumor cells. cKDH-8/11 is a sub-clone isolated from the primary culture of KDH-8 tumor cells by limiting dilution.[7] These cell lines have properties similar to those of the parent KDH-8 *in vivo* and have been maintained in a continuous *in vitro* culture in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS).

Reagents

ONO-4007 (sodium 2-deoxy-2-[3s-(9-phenylnonayloxy) tetradecanoyl]-amino-3-*O*-(9-phenylnona-nonyl)-D-glucopyranose 4-sulfate) was kindly provided by ONO Pharmaceutical (Osaka, Japan). Human natural TNF-α was a gift from Department of Neurosurgery, Hokkaido University School of Medicine.

In vivo antitumor effects

On day 0, KDH-8/YK and cKDH-8/11 cells (1×10^5, respectively) were transplanted s.c. in WKAH rats.

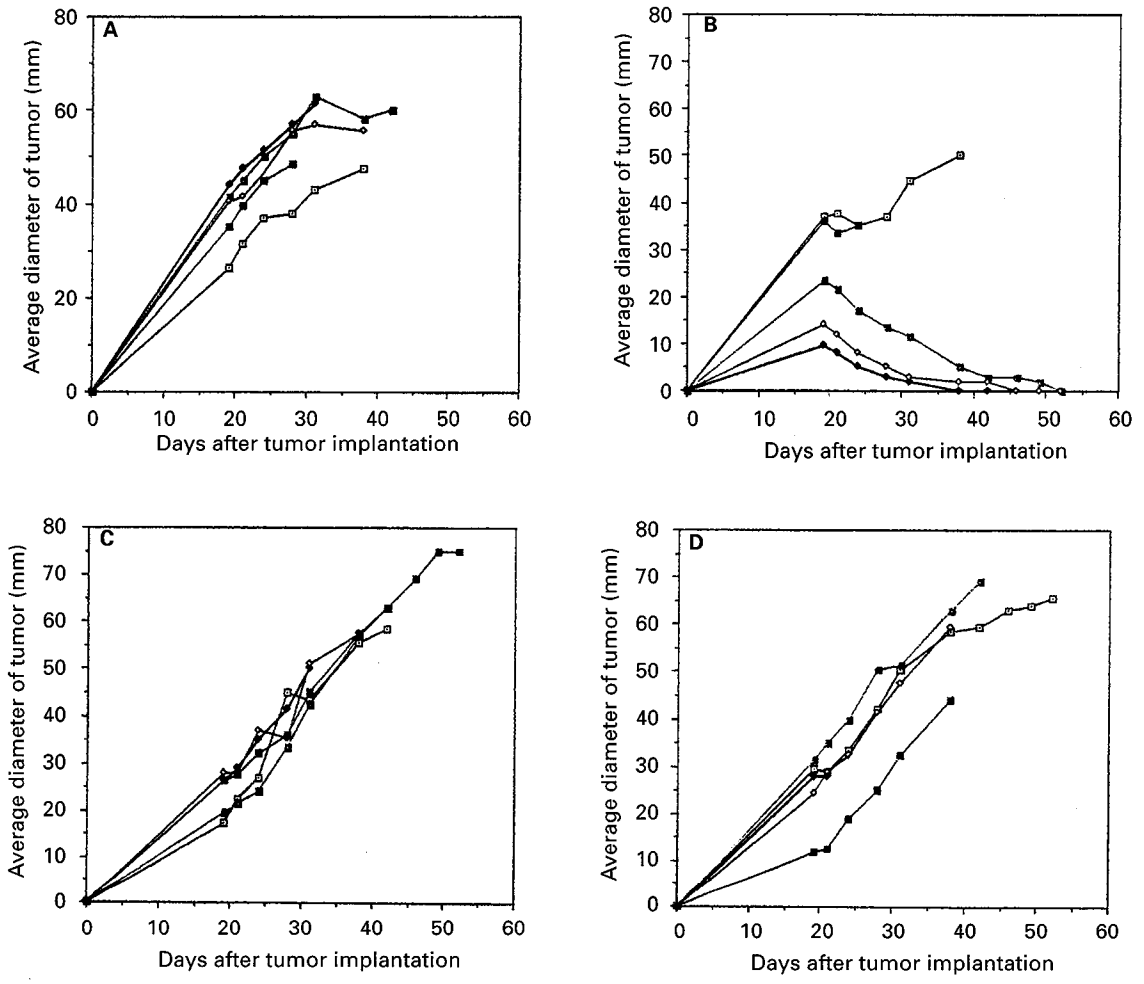

Figure 1. Individual growth curves of rat hepatoma KDH-8/YK and cKDH-8/11 in WKAH rats treated with or without ONO-4007 *in vivo*. (A) Saline-treated KDH-8/YK-bearing rats (saline i.v.; $n=5$). (B) ONO-4007-treated KDH-8/YK-bearing rats (2.5 mg/kg i.v.; $n=5$). (C) Saline-treated cKDH-8/11-bearing rats (saline i.v.; $n=5$). (D) ONO-4007-treated cKDH-8/11-bearing rats (2.5 mg/kg i.v.; $n=5$).

On days 7, 14 and 21, ONO-4007 (2.5 mg/kg) or saline was administered i.v. to the rats. Tumor size was measured every other day. The rats were observed up to 60 days after tumor transplantation when tumors were no longer detected in any of the surviving rats.

Assay for cytotoxicity

KDH-8/YK and cKDH-8/11 cells (1×10^4, respectively) were plated in 100 µl of RPMI 1640 medium supplemented with 10% FBS in 96-well flat-bottomed microplates and incubated in a CO_2 incubator. After 24 h natural human TNF-α was added to the plates. The cultures were incubated for 48 h. Assay for cytotoxicity was performed by a colorimetric crystal violet staining method.[8]

Results

In vitro cytotoxic effects of TNF-α on KDH-8/YK and cKDH-8/11 cells

Table 1 shows that KDH-8/YK cells were sensitive to TNF-α. TNF-α inhibited KDH-8/YK cell growth. However, cKDH-8/11 cells were resistant against TNF-α.

Therapeutic effects of ONO-4007 on KDH-8/YK and cKDH-8/11 in WKAH rats

Figure 1 shows individual growth curves of KDH-8/YK and cKDH-8/11 tumor cells in WKAH rats treated with or without ONO-4007. Although no untreated KDH-8/YK-bearing rats survived more than 55 days, some ONO-4007-treated KDH-8/YK-bearing rats survived up to 60 days. However, the treatment with ONO-4007 had no effect on the growth of cKDH-8/11 in WKAH rats. Severe side effects were not observed. Table 2 shows the lethal growth incidence of KDH-8/YK and cKDH-8/11 cells in rats treated with or without ONO-4007. Sixty percent of KDH-8/YK-bearing rats treated with ONO-4007 survived.

Table 1. Effects of natural human TNF-α on *in vitro* growth of cKDH-8/11 and KDH-8/YK cells

Concentration of TNF-α (U/ml)	cKDH-8/11 (% growth inhibition)	KDH-8/YK (% growth inhibition)
100	0.7	62.4
50	0.4	57.6
25	6.9	49.1
12.5	0	35.6
6.3	10.4	23.2
3.2	8.3	12.0
1.6	2.5	4.6
0.8	3.5	1.3

These data were obtained by a colorimetric crystal violet stain assay. Cells (1×10^4) were plated in 100 µl of medium in 96-well flat-bottomed microplates and incubated for 24 h. The cells were then treated with natural human TNF-α for 48 h.

Table 2. Effects of ONO-4007 on hepatocellular carcinoma cKDH-8/11 and KDH-8/YK cells in WKAH rats[a]

Tumor	Treated with ONO-4007 (mg/kg)[b]	Cured/ treated (%)	Mean survival time (days ± SD)
cKDH-8/11	no	0/5 (0)	38.2 ± 4.8
	yes	0/5 (0)	34.0 ± 6.0
KDH-8/YK	no	0/5 (0)	43.6 ± 6.3
	yes	3/5 (60)	40.2 ± 10.1

[a]cKDH-8/11 and KDH-8/YK cells (1×10^5) were transplanted s.c. in WKAH rats on day 0.
[b]ONO-4007 (2.5 mg/kg) was administered i.v. to the rats on days 7,14 and 21.

Discussion

The results of the present study indicate that a new synthetic lipid A derivative, ONO-4007, had significantly potent therapeutic effects on rats bearing TNF-sensitive KDH-8/YK cells, but not on rats bearing TNF-resistant cKDH-8/11 cells. TNF-α was produced in the cKDH-8/11 tumor tissues as much as in those of KDH-8/YK (data not shown). On the basis of these findings, we suggest that the intratumoral TNF-α plays a major role in the antitumor mechanisms of ONO-4007 against TNF-sensitive tumors.

We could not elucidate whether or not the TNF-α produced in tumor tissues was a direct killing factor to eliminate all tumor cells *in situ*. In general, spontaneous tumors that develop in human are heterogeneous. Consequently in tumor tissue, some tumor cells are TNF sensitive and others are TNF resistant.[9,10] If the therapeutic effect of ONO-4007 is exerted only by the direct killing ability of TNF-α induced by ONO-4007, it is difficult to cure the tumor bearer completely. However, tumor cells killed by TNF-α were presumably relative to the trigger of immunoreaction against TNF-α-resistant tumor cells. Thus, we must study whether ONO-4007 is effective for treating tumor bearers of both KDH-8/YK and cKDH-8/11.

In conclusion, our present study has demonstrated that a new synthetic lipid A derivative, ONO-4007, had a therapeutic effect against a TNF-sensitive tumor cell line, but not against a TNF-resistant tumor cell line and

that TNF-α induced in tumor tissues by ONO-4007 was important in the biotherapy of TNF-α-sensitive tumor bearers.

Acknowledgments

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