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The mechanism of locally enhanced production of tumor necrosis factor-α in tumor tissues by the administration of a new synthetic lipid A analog, ONO-4007, in hepatoma-bearing rats

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ONO-4007 is a new synthetic lipid A analog with low endotoxic activities. We previously found that ONO-4007 induced the production of tumor necrosis factor (TNF)-α in rat hepatoma KDH-8 tumor tissues and brought about the regression of transplanted KDH-8 cells. By contrast, ONO-4007 did not induce TNF-α production in spleens and sera 90 min after treatment. In the present study we attempted to elucidate how ONO-4007 induces TNF-α production in tumor tissues locally. We found that extracellular matrix including gelatin, fibronectin and Matrigel® did not induce TNF-α production in splenocytes treated with ONO-4007 *in vitro*. However, splenocytes co-cultured with cKDH-8/11 tumor cells in the presence of ONO-4007 produced more TNF-α than splenocytes cultured by themselves in the presence of ONO-4007. The stimulation of cKDH-8/11 cells in the presence of ONO-4007 for splenocytes to produce TNF-α depended on the type of contact between the cells. The cKDH-8/11 cells fixed in formalin were not able to induce TNF-α production of splenocytes even in the presence of ONO-4007. However, syngeneic fibrosarcoma cell line KMT-17/A3, allogeneic hepatocellular carcinoma cell line LDH and rat lung endotherial cell line RLE induced TNF-α production in splenocytes, but their stimulation was weaker than that of cKDH-8/11. The soluble form of the cKDH-8/11 cell membrane did not stimulate splenocytes to produce TNF-α in the presence of ONO-4007. cKDH-8/11 cells did not stimulate the splenocytes devoid of macrophages to produce TNF-α in the presence of ONO-4007.

Key words: Hepatocellular carcinoma, lipid A, ONO-4007, tumor necrosis factor.

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Introduction

Lipopolysaccharide (LPS) in a Gram-negative bacterial envelope is a potent stimulator of many early events in macrophage activation, such as production and secretion of interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α.[1,2] 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 which are less harmful to hosts, but they have not been equipped with potent immunopharmacological activities combined with low toxicity.[3–5] A novel lipid A derivative, ONO-4007, has low toxicity of less than 1/1000 that of natural *Escherichia coli* LPS.[6] *In vivo* treatment with i.v. administration of ONO-4007 brought about therapeutic effects in rats inoculated with leukemic cells[7] and hepatoma cells,[8] without severe side effects.

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.[9] Production of a large amount of TNF-α in tumor tissues can cause inhibition of tumor growth.[10,11] We reported that ONO-4007 markedly induced production of TNF-α in rat hepatocellular carcinoma KDH-8 tumor tissues, but not in sera and spleens in hepatoma-bearing rats 90 min after administration.[8] Here, we describe a possible mechanism responsible for the local production of a large amount of TNF-α in tumor tissues in KDH-8-bearing rats treated with ONO-4007.

Materials and methods

Animals

Female Wistar King Aptekman/Hok (WKAH) rats, 8–12

weeks old, 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*.

Cell lines

KDH-8 is a rat transplantable hepatoma induced by 3′-methyl-4-dimethylaminoazo-benzene in a WKAH rat and has been maintained *in vivo* by i.p. passage every 5 days. The cKDH-8/11 is a sub-clone isolated from the primary culture of KDH-8 tumor cells by limiting dilution and has properties similar to those of the parent *in vivo* line. cKDH-8/11 cells have been maintained in a continual *in vitro* culture in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS).[12] 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 sub-clone of the *in vitro* KMT-17 parental line and has properties similar to those of the parent *in vivo* line. KMT-17/A3 cells were maintained in a continual *in vitro* culture in RPMI 1640 medium supplemented with 10% FBS.[13] LDH is a transplantable hepatoma line induced by 3′-methyl-4-dimethylaminoazo-benzene in a Long Evans Cinnamon (LEC) rat and maintained in a continual *in vitro* culture in Dulbecco's modified Eagle's medium (DMEM; Nissui) supplemented with 10% FBS. RLE is a rat lung endotherial cell line established from Fisher 344 rats. Those cells were maintained continually *in vitro* in 1.0% gelatin-coated plastic tissue culture plates (Corning, USA) containing a 1:1 ratio of DMEM and Ham's nutrient mixture F12 (Nissui) medium supplemented with 15% FBS.[14]

Splenocytes

The rats were killed and the spleens were removed aseptically. Single-cell suspensions were obtained by gently teasing the spleen in a loose-fitting ground-glass homogenizer and passing it through sterile metal mesh. Erythrocytes were removed by hypo-osmotic shock and the remaining cells were washed three times with cold RPMI medium supplemented with 2% FBS.[13]

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). 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.

Preparation of culture supernatant of the splenocytes cultured on culture plates coated with extracellular matrix (ECM)

Splenocytes (1×10^6/ml) were seeded in 2 ml of 10% FBS RPMI in 24-well culture plates (Corning) coated with 1.0% gelatin (Wako, Osaka, Japan), 50 μg/ml bovine plasma fibronectin (Gibco/BRL, Gaithersburg, MD) and 250 ng/μl Matrigel™ matrix (Becton Dickinson Labware, Bedford, MA), and cultured with ONO-4007 (5 μg/ml) for 24 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay.

Preparation of culture supernatant of the splenocytes co-cultured with cKDH-8/11, KMT-17/A3, LDH and RLE cells

cKDH/11, KMT-17/A3, LDH and RLE cells (1×10^6/ml) were seeded with or without splenocytes, respectively, in 2 ml of 10% FBS RPMI in 24-well culture plates and cultured in the presence of ONO-4007 (5 μg/ml) for 6 or 24 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay.

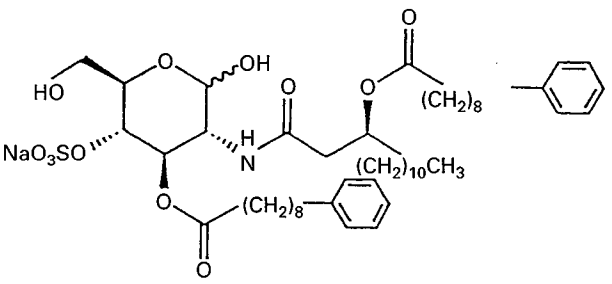

Figure 1. Chemical structure of ONO-4007.

Preparation of culture supernatant of the splenocytes co-cultured with mitomycin C (MMC)- or formalin-treated cKDH-8/11 cells or their membrane fraction

Before being co-cultured with the splenocytes, cKDH-8/11 cells were either cultured in 10% FBS RPMI with MMC (Kyowa Hakko Kogyo, Tokyo, Japan; 100 μg/ml) for 1 h in a CO_2 incubator and washed three times with 2% FBS RPMI or fixed in 10% formalin for 15 min and washed three times with 2% FBS RPMI. The cells (1×10^6/ml) were seeded with or without the splenocytes in 2 ml of 10% FBS RPMI in 24-well culture plates and cultured in the presence of ONO-4007 (5 μg/ml) for 24 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay.

n-Butyl alcohol extraction was performed using a modification of the method of Sato and Kikuchi.[15] The glycoprotein was extracted from 10^7 cKDH-8/11 cells with 1.25% *n*-butyl alcohol for 15 min providing about 200 μg/ml of protein without cellular lysis. After sonication, these samples were centrifuged at 100 000 *g* for 1 h and then samples of the supernatant were collected. Splenocytes (1×10^6/ml) were cultured in 2 ml of 10% FBS RPMI in 24-well culture plates in the presence of cKDH-8/11-membrane with ONO-4007 (5 μg/ml) for 6 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay.

Preparation of culture supernatant of the splenocytes contacted with or without cKDH-8/11 cells in the Transwell™

cKDH/11 cells (1×10^6) were seeded in 100 μl of 10% FBS RPMI in upper chambers and 1×10^6 splenocytes were seeded in 600 μl of 10% FBS RPMI in lower chambers of a 0.4 μm pore size Transwell™ (Costar, Cambridge, MA). The cells were cultured in the presence of ONO-4007 (5 μg/ml) for 24 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for the TNF-α bioassay.

Bioassay for TNF-α

The TNF-α assay employed in this experiment was the 1 day assay using L-929, a TNF-α-sensitive murine fibroblast cell line, in the presence of 0.2 μg/ml actinomycin D.[16]

Depletion of T cells and macrophages from splenocytes

T cells and macrophages were depleted from splenocytes by using Dynabeads M-450 rat anti-mouse IgG2a (Dynal, Oslo, Norway), mouse anti-rat macrophage monoclonal antibody (CL042A; Cedarlane, Ontario, Canada) and mouse anti-rat pan T lymphocyte monoclonal antibody (CL052A; Cedarlane). In brief, fresh splenocytes were incubated with CL042A and/or CL052A for 30 min at 4°C. The cells were centrifugated at 200 *g* for 10 min and the supernatant was discarded. The cells were resuspended and washed twice with Hank's balanced salt solution (HBSS), pH 7.4, to remove all unbound antibodies. The target cells treated with CL042A and/or CL052A were isolated by adding Dynabeads M-450 rat anti-mouse

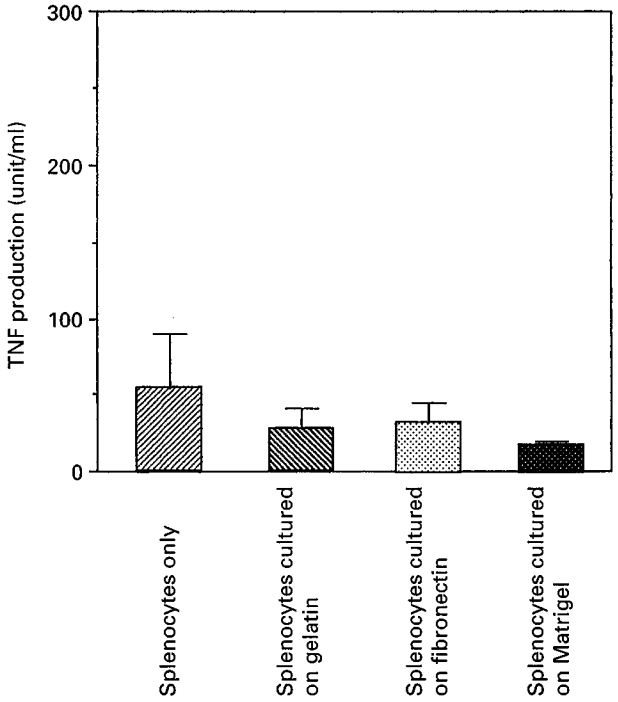

Figure 2. TNF-α production in the supernatant of splenocytes cultured on ECM in the presence of ONO-4007 *in vitro*. Splenocytes (1×10^6/ml) were seeded in 2 ml of 10% FBS RPMI in 24-well culture plates coated with 1.0% gelatin, 50 μg/ml of bovine plasma fibronectin and 250 ng/μl of Matrigel™ matrix, respectively, and cultured with ONO-4007 (5 μg/ml) for 24 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay.

IgG2a, incubated and separated with a Dynal Magnetic Particle Concentrator.[17,18]

Statistical analysis

Statistical determinations, where applied, were calculated using Student's *t*-test.

Results

Effects of ECM on TNF-α production of the splenocytes *in vitro*

Whereas production of TNF-α was observed in the tumor tissues of hepatoma-bearing rats treated with ONO-4007, this was not detected in the sera and the spleens of the animals.[8] We observed more tumor-infiltrating cells in the tumor tissues of rats treated with ONO-4007 than in those of untreated rats (data not shown). The tumor infiltrating cells moved into the tumor tissues through endothelial cells and ECMs. Figure 2 shows the influence of ECMs such as gelatin, fibronectin and Matrigel™ matrix on TNF-α production of the splenocytes treated with ONO-4007 *in vitro*. ECMs did not enhance the TNF-α production of the splenocytes treated with ONO-4007.

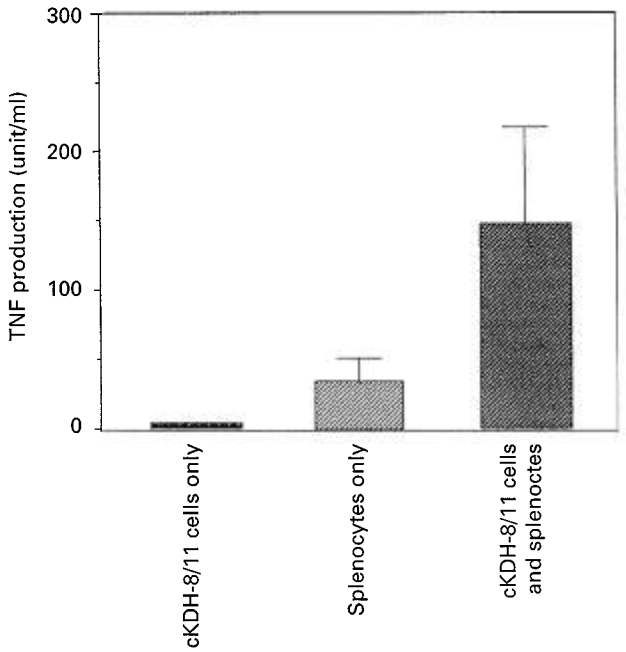

Figure 3. TNF-α production in the supernatant of splenocytes co-cultured with cKDH-8/11 cells in the presence of ONO-4007 *in vitro*. cKDH/11 cells (1×10^6/ml) were seeded with or without splenocytes in 2 ml of 10% FBS RPMI in 24-well culture plates and cultured with ONO-4007 (5 μg/ml) for 24 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay. Splenocytes co-cultured with cKDH-8/11 cells in the presence of ONO-4007 produced significantly more TNF-α than splenocytes alone ($p < 0.05$).

ONO-4007-induced TNF-α production of splenocytes *in vitro* is enhanced by contact with cKDH-8/11 cells

Figure 3 shows that cKDH-8/11 cells did not produce TNF-α themselves upon treatment with ONO-4007,

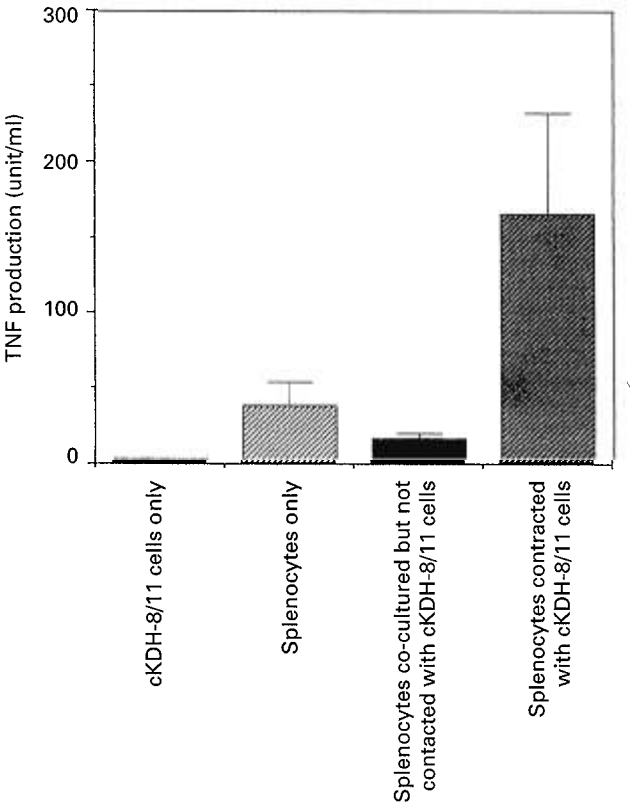

Figure 4. TNF-α production in the supernatant of splenocytes contacted with or without cKDH-8/11 cells in the presence of ONO-4007 *in vitro*. cKDH/11 cells (1×10^6) were seeded in 100 μl of 10% FBS RPMI in upper chambers and 1×10^6 splenocytes were seeded in 600 μl of 10% FBS RPMI in lower chambers of a 0.4 μm pore size Transwell™. They were cultured with ONO-4007 (5 μg/ml) for 24 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay. Splenocytes in contact with cKDH-8/11 cells produced more TNF-α than those co-cultured but not in contact with cKDH-8/11 cells ($p < 0.02$).

but these cells significantly induced TNF-α production of splenocytes treated with ONO-4007 *in vitro* ($p<0.05$).

To elucidate how cKDH-8/11 cells induced TNF-α production of splenocytes, we cultured splenocytes separated from cKDH-8/11 cells using a Transwell® *in vitro*. Figure 4 shows that cKDH-8/11 cells, apart from the splenocytes, could not stimulate each other to produce TNF-α ($p<0.02$), suggesting that the effect of cKDH-8/11 cells is not mediated by humoral factors (e.g. cytokines).

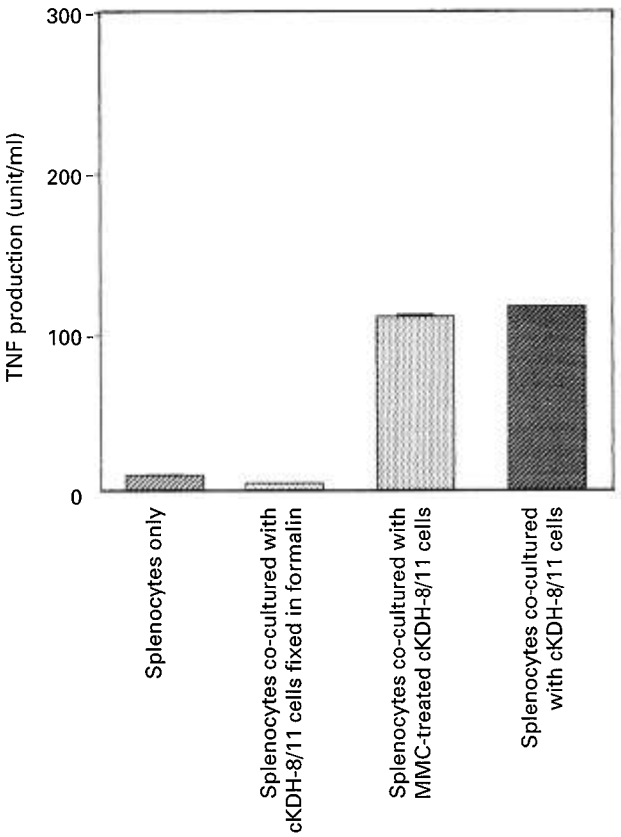

Figure 5. TNF-α production in the supernatant of splenocytes co-cultured with cKDH-8/11 cells treated with mitomycin C or formalin in the presence of ONO-4007 *in vitro*. Before being co-cultured with splenocytes, cKDH-8/11 cells were treated with MMC (100 μg/ml) or 10% formalin for 1 h or 15 min in a CO_2 incubator, and then washed three times with 2% FBS RPMI. The cells (1×10^6/ml) were seeded with or without splenocytes in 2 ml of 10% FBS RPMI in 24-well culture plates and cultured with ONO-4007 (5 μg/ml) for 24 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay. The fixation in formalin inhibited the stimulation of cKDH-8/11 cells for TNF-α production of the splenocytes treated with ONO-4007 ($p<0.001$).

Effects of MMC- and formalin-treated-cKDH-8/11 cells or the soluble form of the cKDH-8/11 cell membrane on TNF-α production by splenocytes *in vitro*

Figure 5 shows that treatment with MMC did not block the stimulation by cKDH-8/11 cells of TNF-α production by splenocytes treated with ONO-4007, whereas fixation of the cells with 10% formalin did block this TNF-α production ($p<0.001$). These results suggest that factors on the surface of cKDH-8/11 cells denatured by fixation with 10% formalin may stimulate TNF-α production by splenocytes treated with ONO-4007. Figure 6 shows that the soluble form of the cKDH-8/11 cell membrane did not stimulate splenocytes to produce TNF-α in the presence of ONO-4007 *in vitro*.

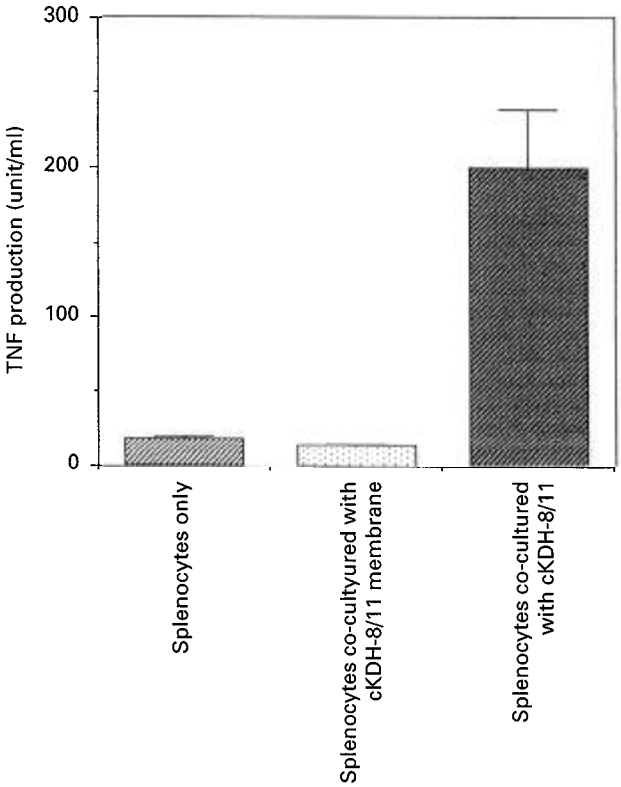

Figure 6. TNF-α production in the supernatant of splenocytes co-cultured with the membrane of cKDH-8/11 cells in the presence of ONO-4007 *in vitro*. Splenocytes (1×10^6/ml) were cultured in 2 ml of 10% FBS RPMI in 24-well culture plates in the presence of the cKDH-8/11 membrane with ONO-4007 (5 μg/ml) for 6 h in a CO_2 incubator. After centrifugation at 20 000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay. The soluble form of the cKDH-8/11 membrane did not induce TNF-α production of the splenocytes ($p<0.005$).

Effects of KMT-17/A3, LDH and RLE cells on TNF-α production by splenocytes *in vitro*

We examined whether syngeneic fibrosarcoma cell line KMT-17/A3, allogeneic hepatocellular carcinoma cell line LDH and rat lung endotherial cell line RLE could also induce TNF-α production of splenocytes. Although every one of them stimulated splenocytes, this stimulation was significantly weaker than that by cKDH-8/11 (Figure 7).

Role of T cells and/or macrophages in the TNF-α production of splenocytes co-cultured with cKDH-8/11 cells *in vitro*

In order to determine which cells in splenocytes play a role in TNF-α production, the influence of T cell depletion or macrophage depletion on splenocytes was examined. Figure 8 shows that cKDH-8/11 cells were not able to stimulate splenocytes deprived of macrophages to produce TNF-α in the presence of ONO-4007 *in vitro*. However, depletion of T cells did not have an effect on TNF-α production of splenocytes co-cultured with cKDH-8/11 cells in the presence of ONO-4007 *in vitro*.

Discussion

The results of this study indicate that the contact between splenocytes and tumor cells is important in their *in vitro* TNF-α production enhanced by a new synthetic lipid A analog (ONO-4007) with low endotoxic activities. This finding corresponds to our previous observation that TNF-α production was

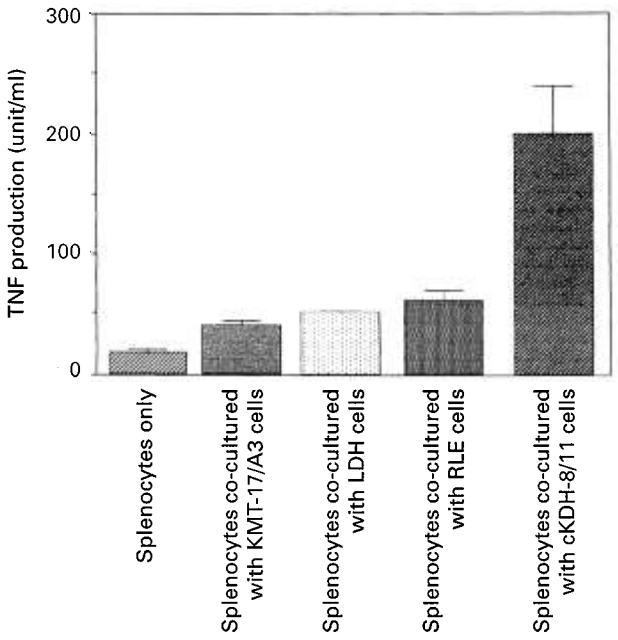

Figure 7. TNF-α production in the supernatant of splenocytes co-cultured with KMT-17/A3, LDH and RLE cells in the presence of ONO-4007 *in vitro*. KMT-17/A3, LDH and RLE cells (1×10^6/ml) were seeded with or without splenocytes, respectively, in 2 ml of 10% FBS RPMI in 24-well culture plates and cultured with ONO-4007 (5 μg/ml) for 6 h in a CO_2 incubator. After centrifugation at 20000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters and used for TNF-α bioassay. Although KMT-17/A3, LDH and RLE cells stimulated splenocytes, the stimulation was significantly weaker than that of cKDH-8/11 ($p<0.01$, $p<0.05$ and $p<0.01$, respectively).

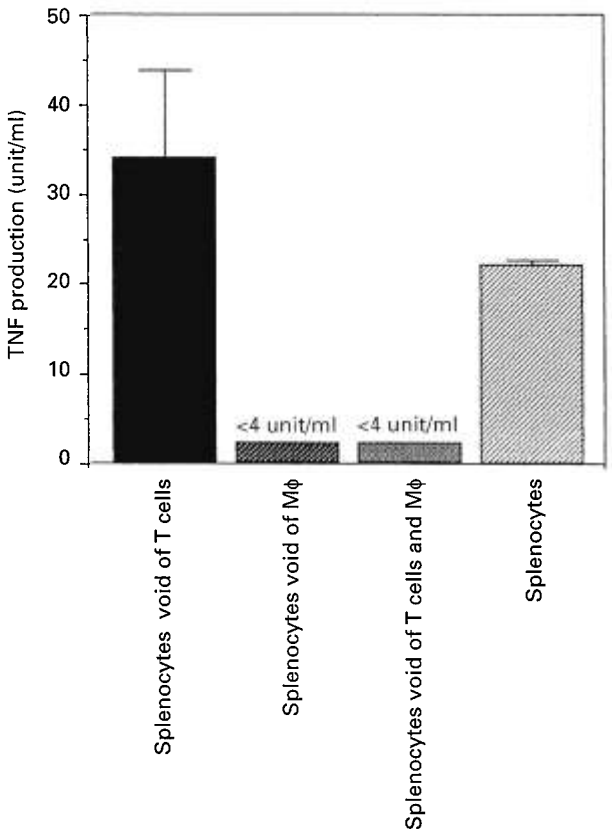

Figure 8. TNF-α production in the supernatant of the splenocytes void of T cells and/or macrophages co-cultured with cKDH-8/11 cells in the presence of ONO-4007 *in vitro*. Splenocytes (1×10^6/ml) devoid of T cells and/or macrophages were cultured in 2 ml of 10% FBS RPMI in 24-well culture plates in the presence of cKDH-8/11 cells with ONO-4007 (5 μg/ml) for 6 h in a CO_2 incubator. After centrifugation at 20000 *g* for 60 min, the supernatant was collected and passed through 0.45 μm pore size filters to be used for TNF-α bioassay.

enhanced only in tumor tissue but not in spleens or in sera of KDH-8 hepatoma-bearing rats treated with ONO-4007[8]. Contact of splenocytes with intact tumor cells seemed to be nearly indispensable for TNF-α production *in vitro*, since separation of splenocytes and tumor cells by a millipore filter (Figure 4), formaldehyde-fixed tumor cells (Figure 5) or the tumor cell membrane fraction by itself (Figure 6) did not induce TNF-α production with ONO-4007 *in vitro*. We also considered the possibility that ECM in tumor tissue might stimulate tumor-infiltrating lymphoid cells and play a role in the enhancement of TNF-α production. However, ECM did not help splenocytes to produce TNF-α *in vitro* (Figure 2).

Although stimulation with syngeneic fibrosarcoma cell line KMT-17/A3, allogeneic hepatoma cell line LDH or rat lung endotherial cell line RLE also enhanced TNF-α production of splenocytes with ONO-4007 *in vitro*, it was much weaker than that with intact or MMC-treated cKDH-8/11 cells (Figures 5 and 7). We speculate, therefore, that cell membrane molecules of cKDH-8/11 cells might be responsible for inducing TNF-α production *in vitro*. However, we could not identify the molecule, since butylate-extracted membrane did not enhance it (Figure 6). We also observed that not only syngeneic or allogeneic tumor cells of various cellular origins, but also normal lung endotherial cells stimulated the splenocytes to produce TNF-α. These findings suggest that the signals which enable ONO-4007 to induce TNF-α production of splenocytes may be on the surface of normal cells, as well as on that of tumor cells.

Regarding the cell population producing TNF-α, it is reasonable to conclude that the macrophages in spleen produce TNF-α after contacting with tumor cells and ONO-4007. In fact, macrophage-depleted splenocytes did not produce TNF-α, whereas depletion of T lymphocytes did not affect the TNF-α production of splenocytes (Figure 8). In the present experiment, we could not rule out the role of T lymphocytes in the TNF-α production of macrophages, as the depletion of T lymphocytes was not complete. In the interaction between tumor cells and host immunocompetent cells, adhesion molecules such LFA-1 and ICAM-1 are important for their functions, which require binding and adhesion.[19,20] However, we observed that anti-LFA-1 or anti-ICAM-1 antibodies could not inhibit the induction of TNF-α production of splenocytes by their contact with cKDH-8/11 cells (data not shown). Further studies are required to investigate this point.

In conclusion, the findings in this study may provide some explanation for the high local TNF-α production in tumor tissues following administration of ONO-4007 and a clue to elucidate the signals responsible for the production of TNF-α in these tumor tissues.

Acknowledgments

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