Abstract. Toll-like receptors (TLRs) are important molecules that stimulate the innate immunity in order to eradicate microbial pathogens, after which the adaptive immunity emerges. The involvement of TLRs in the action mechanism of OK-432, a bacterial preparation, was investigated in the locoregional treatment of malignant ascites from gastric cancer. The expression of TLRs in ascites cells was analyzed using reverse-transcription polymerase chain reaction specific for TLRs and by flow cytometry using anti-TLR2, -TLR4, -CD4, -CD8, and -CD11c antibodies. These measurements were compared with the locoregional response of OK-432 immunotherapy for malignant ascites, as well as TNF-α producing potential, which was measured by ELISA, of ascites cells stimulated in vitro with OK-432. It was observed that OK-432 immunotherapy for malignant ascites showed 8 positive (67%) and 4 negative responses with the tolerable adverse effects of fever elevation and abdominal pain. The TNF-α production of ascites cells by in vitro OK-432 stimulation was significantly higher in responder patients than in non-responders. The clinical responses were correlated with the expression of the TLR4 gene of ascites cells. The TNF-α producing potential of ascites cells by in vitro OK-432 stimulation was dependent on the existence of a CD11c+TLR-4+ cell population in ascites cells. OK-432 was highly stimulatory for TNF-α production of ascites cells compared with other biological response modifiers of PSK and LEM. These results suggest that TLR-4 expression on ascites cells of a macrophage lineage is essential for ascites cells to produce TNF-α in relation to OK-432 stimulation and for subsequent positive clinical responses in locoregional immunotherapy using OK-432 for malignant ascites from gastric cancer.

The Toll-like receptor (TLR) recognizes a broad range of microbial pathogens such as bacteria and viruses, triggering inflammatory and antiviral responses and dendritic cell maturation (1, 2). This recognition plays an instructive role in innate immune responses against microbial pathogens, as well as in the subsequent induction of adaptive immune responses, resulting in the eradication of invading pathogens (1, 2). Many TLRs have been identified as making up a TLR family. Each of these TLRs has been involved in the recognition of specific molecular patterns found in ligands, including lipids, proteins and nucleic acids (3). TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for lipopolysaccharide (LPS). TLR5 recognizes flagellin. TLR11 recognizes profilin-like protein. TLR3, 7(8) and 9 are found to recognize nucleic acids, such as double-stranded, or single-stranded RNA or DNA, respectively (3). As a result of TLR stimulations by cognate ligands, pro-inflammatory responses, including cytokines such as TNF, IL-6, IL-12, and co-stimulatory molecules, are induced (1-3).

Biological response modifiers (BRMs), including a bacterial preparation, protein-bound polysaccharide extracted from plants, synthetic proteins, and lipids have been introduced into immunotherapy for cancer during the last quarter century (4, 5). It may be important to clarify the relationship between TLRs and the action mechanisms.
of BRMs, considering that such BRMs may stimulate TLRs for positive clinical responses. There are several reports demonstrating the relevance between therapeutic agents and TLR expression. *Mycobacterium bovis* bacille Calmette-Guerin (BCG) has been found to require TLR2 and TLR4 expression for its biological activities (6, 7). We have been engaged in cancer immunotherapy using a streptococcal preparation, OK-432, and have shown that it induces an acute inflammation-like locoregional response based on the nature of its microbial preparation (8-10). In this paper, we attempted to further address the mode of action of OK-432 in view of a relationship between TLR status and the responses from OK-432 immunotherapy for locoregional management of malignant ascites in gastric cancer patients.

**Materials and Methods**

**Patients and locoregional immunotherapy using OK-432.** Twelve gastric cancer patients with cytologically proven malignant ascites were enrolled in the study. The subjects consisted of 9 males and 3 females (mean age of 64 and 61 years, respectively), who had measurable ascites on sonographic examination and computed tomographic (CT) scans, and had an Eastern Cooperative Oncology Group performance status of 0 to 3. Written informed consent was obtained from each patient before treatment. The ascites may have been removed by paracentesis before treatment, and the host-oriented dose of OK-432 (10) was administered thereafter. OK-432 administration was repeated once on day 8 when no decrease of ascites was observed. A 5-fluorouracil-based combination chemotherapy had failed to reduce the ascites in all of our subjects. Clinical responses were assessed on days 15 and 43 by cytological and sonographic examination together with CT scans. We then assigned our patients to one of two groups: responders, who showed a disappearance or decrease of ascites with negative cytology lasting for more than 1 month after the treatment; and non-responders, who had stable or increasing ascites even after the treatment (8-10).

**Preparation of locoregional ascites cells.** Heparinized ascites were obtained by paracentesis before treatment and ascites cells were pelleted. Cells were resuspended in RPMI-1640 medium and layered on 75/100% Ficoll-Conray gradient. After centrifugation at 400 xg for 30 min, autologous tumor cells were collected from a 75% interface and mononuclear cells from a 100% interface. Mononuclear cells in aliquots were pelleted and frozen in liquid nitrogen for RNA extraction. Mononuclear cells were also washed 3 times, resuspended in RPMI-1640 medium supplemented with 2% heat-inactivated autologous serum, and subjected to further experiments.

**TLR mRNA expression.** Reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze TLR mRNA expression. In brief, total RNA was extracted from 5x10^5 ascites cells.
cells and reverse-transcribed with random hexamer, as described previously (11). Aliquots of the cDNA were amplified with PCR, using TLR-specific oligonucleotides on a Perkin-Elmer Cetus thermal cycler (Perkin Elmer, Norwalk, CT, USA). The primer sequences used are shown in Table I (12). The reaction was carried out under the following conditions: 3-min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. After amplification, 8 µl of the reaction mixture was removed and analyzed by means of electrophoresis through 2.0% agarose gels in Tris-borate-EDTA buffer, and the gels were then stained with ethidium bromide.

Cytokine assay. Mononuclear cells (1x10⁶/ml) were stimulated either with 0.1 KE/ml of OK-432 (Chugai Pharmaceutical Company, Tokyo, Japan), 100 µg/ml of polysaccharide-K (PSK, Kureha Chem. Indust., Tokyo, Japan), or 100 µg/ml of Lentinus Edodes Mycelia (LEM, Kobayashi Pharm. Kyoto, Japan). Incubation was performed at 37°C in 5% CO₂ incubator for 24 h. Supernatants were collected and the production of TNF-α was measured using ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Flow cytometry. Mononuclear cells (5x10⁵) were incubated either with anti-TLR2 or -TLR4 antibody (Monosan, USA) at 4°C for 30 min. Cells were washed and stained with phycoerythrin-labelled rat anti-mouse Igκ light chain antibody (BD Biosciences, Tokyo, Japan). Cells were then washed again 3 times and double-stained with fluorescein isothiocyanate-labeled anti-CD4, -CD8, or -CD11c antibodies (Becton Dickinson, San Jose, CA, USA). Finally, the cells were washed twice and resuspended in phosphate buffered saline, and flow cytometric analysis was performed using a Cytoron (Ortho Diagnostic Systems, USA).

Statistical analysis. Statistical analysis was conducted using the χ² test, Student’s t-test or regression analysis using StatView software (Version 5) on a Macintosh computer.

Results

Clinical responses of locoregional immunotherapy for malignant ascites using OK-432. The clinical efficacy of locoregional immunotherapy using OK-432 for malignant ascites was assessed in serial gastric cancer patients with malignant ascites. A positive response was observed in 8 (67%) and a negative response in 4 (33%) of 12 patients treated. Adverse effects were fever elevation in 12 (100%) and abdominal dull pain in 4 (33%) out of 12 patients enrolled, which were well-tolerated with non-steroidal anti-inflammatory medications.

Relationship between locoregional responses to OK-432 immunotherapy and TNF-α production by ascites cells. The TNF-α production potential of ascites cells was assessed (Figure 1). Ascites cells produced 53 to 8370 pg/ml TNF-α with in vitro OK-432 stimulation. Regarding the clinical responses, ascites cells from responder patients produced an overall mean value of

<table>
<thead>
<tr>
<th>Expression</th>
<th>Responder (n=8)</th>
<th>Non-responder (n=4)</th>
<th>p</th>
</tr>
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<tr>
<td>TLR1</td>
<td>7</td>
<td>3</td>
<td>N.S.</td>
</tr>
<tr>
<td>TLR2</td>
<td>8</td>
<td>4</td>
<td>N.S.</td>
</tr>
<tr>
<td>TLR3</td>
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<td>8</td>
<td>4</td>
<td>N.S.</td>
</tr>
<tr>
<td>TLR6</td>
<td>7</td>
<td>3</td>
<td>N.S.</td>
</tr>
<tr>
<td>TLR7</td>
<td>4</td>
<td>3</td>
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</tr>
<tr>
<td>TLR8</td>
<td>6</td>
<td>4</td>
<td>N.S.</td>
</tr>
<tr>
<td>TLR9</td>
<td>3</td>
<td>3</td>
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</tr>
<tr>
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</tr>
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<tr>
<td>GAPDH</td>
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Locoregional immunotherapy of malignant ascites using OK-432 was performed and clinical responses were assessed as described in Materials and Methods. Ascites cells were collected and subjected to RT-PCR analysis specific for TLR expressions. N.S.: not significant.

Table II. Relationship between locoregional responses induced by OK-432 immunotherapy of malignant ascites and the expression of TLR mRNAs in ascites cells.

Figure 2. Diverse expression of TLRs in ascites cells. Ascites cells were collected and mRNA was extracted. RT-PCR analysis specific for TLRs was performed. Patient 1 and 2 showed a positive and a negative band for TLR4 expression, respectively.
3974±3236 pg/ml standard deviation ranging from 141 to 8370 pg/ml TNF-α, whilst those from non-responders gave only 83±31 pg/ml ranging from 53 to 120 pg/ml TNF-α. There was a significant difference between these values (p<0.05).

**Relationship between locoregional responses to OK-432 immunotherapy and expression of TLR mRNAs on effusion cells.** The TLR mRNA expression of ascites cells was measured using RT-PCR and the relationship between the TLR mRNA expression and clinical responses of OK-432 immunotherapy was analyzed. A diverse expression of TLR1 to TLR10 mRNAs was observed in the ascites cells tested (Figure 2). Patient 1 and 2 showed a positive and a negative band for the TLR4 mRNA expression, respectively. Regarding the clinical responses to OK-432 immunotherapy, TLR4 mRNA expression was detectable in 7 of 8 responders but not in any of the 4 non-responders (Table II). There was a significant difference in TLR4 mRNA expression between responders and non-responders (p<0.05). However, there were no differences among other TLR expressions between responders and non-responders.

**Phenotypic analysis of TLR-expression of ascites cells.** Two-color flow cytometry was performed to explore the phenotype expressing TLR2 or TLR4 molecules (Figure 3). TLR2 and TLR4 molecules were expressed at very low levels on CD4+ or CD8+ ascites cells (data not shown). The TLR4 molecule was diversely expressed on CD11c+ cells. Representative cytograms are shown in Figure 3, where CD11c+TLR-4+ cells comprised 1.4% of the ascites cells from one patient and 29.1% of those from another patient. The former cells produced only 95 pg/ml of TNF-α while the latter produced 8370 pg/ml of TNF-α in response to in vitro OK-432 stimulation.

**Figure 3.** Two-color flow cytometry for CD11c+TLR4+ cells and TNF-α production by effusion cells with in vitro OK-432 stimulation. Ascites cells were collected and subjected to two-color flow cytometry using anti-TLR4 and anti-CD11c antibodies. Cells were also stimulated in vitro with OK-432 for 24 hours, and TNF-α production was measured with ELISA.

**Figure 4.** Relationship between the CD11c+TLR4+ cell population and the TNF-α production by effusion cells with in vitro OK-432 stimulation. Ascites cells were collected and subjected to two-color flow cytometry using anti-TLR4 and anti-CD11c antibodies. Cells were also stimulated in vitro with OK-432 for 24 h, and TNF-α production was measured with ELISA. The relationship between the CD11c+TLR4+ cell population and the TNF-α production by effusion cells was analyzed by regression analysis.
Relationship between CD11c+TLR-4+ cell population and TNF-α production potential of ascites cells by OK-432 stimulation. We analyzed the relationship between the CD11c+TLR-4+ cell population and the TNF-α production potential in response to *in vitro* OK-432 stimulation (Figure 4). As suspected from the profiling of Figure 3, ascites cells that had more CD11c+TLR-4+ cell populations appeared to produce more TNF-α on *in vitro* OK-432 stimulation. There was a significant positive correlation between CD11c+TLR-4+ cell populations and TNF-α production induced by *in vitro* OK-432 stimulation (*p* = 0.0004).

Comparison of TNF-α production of ascites cells on OK-432, PSK, and LEM stimulation. Finally, we compared the TNF-α production potential of ascites cells when stimulated *in vitro* with OK-432 or with PSK or LEM, which are known biological response modifiers (Figure 5). PSK stimulated ascites cells to produce 50 to 200 pg/ml of TNF-α whilst LEM stimulated the production of 50 to 1150 pg/ml of TNF-α. There were no significant correlations in TNF-α production between stimulations by OK-432 and PSK, nor between stimulations by OK-432 and LEM. There were also no correlations between CD11c+TLR-4+ cell populations and the TNF-α production of ascites cells when stimulated with PSK or LEM (data not shown).

Discussion

It has been reported that locoregional administration of OK-432 is effective for managing malignant ascites at approximate response rates of 53 to 70% (8-10, 13). In this study, the efficacy of the locoregional administration of OK-432 was 67% for treating malignant ascites from gastric cancer, confirming the previous efficacy. Although the prognosis of these patients with malignant ascites is extremely poor, the reduction or disappearance of ascites is important for patients’ quality of life (8-10). The locoregional administration of OK-432 is a simple, easy and effective treatment modality for managing malignant ascites from gastric cancer.

The mode of action of OK-432 in cancer treatment have been investigated aggressively. Katano and Torisu (13) reported an increasing number of intraperitoneal neutrophils after OK-432 injection, and these neutrophils had a cytostatic effect on ascites-derived tumor cells *in vitro*. Fujimoto *et al.* (14) have demonstrated a property of OK-432 as a potent T-helper type 1 (Th-1) cytokine inducer. We have previously reported modes of action of OK-432 for malignant ascites. For example, OK-432 induces a serial cellular infiltration of granulocytes, macrophages and lymphocytes into ascites; also, OK-432-induced lymphocytes are CD4+, possess autologous tumor-killing activity, and secrete T-helper type (Th)-1 cytokines, including TNF-α (8, 9). These demonstrations and the nature of OK-432 as a bacterial preparation have motivated us to investigate the involvement of TLRs in the action of OK-432. In this study, ascites cells from patients who responded to OK-432 immunotherapy had greater potential to produce TNF-α against *in vitro* OK-432 stimulation than did ascites cells from non-responder patients, indicating a close relationship of TNF-α to positive responses in the locoregional immunotherapy of malignant ascites using OK-432. Although the expressions of TLR1 to TLR10 genes in ascites cells were highly diverse among the patients with the malignant ascites studied, the TLR4 gene expression correlated significantly with positive clinical responses of locoregional immunotherapy for malignant ascites using OK-432. TLR4 gene was expressed in ascites cells from most of the responder patients in our study, while, in contrast, it was
not expressed at all in the ascites cells from non-responders. Moreover, the CD11c+TLR4+ cell population correlated well with the TNF-α production potential of ascites cells, suggesting the importance of TLR4 expression on ascites cells of macrophage/monocyte lineage, including dendritic cells, in our OK-432 immunotherapy. TNF-α has been reported to be a Th-1 cytokine (15) and to play a pivotal role in innate immunity (16). TLR4 has been reported to be stimulated by LPS, cytokine (15) and to play a pivotal role in innate immunity. TNF-α has been reported to be a Th-1 lineage, including dendritic cells, in our OK-432 immunotherapy. Moreover, the CD11c+TLR4+ cell has not been effective, in contrast, for those who had positive IL-10 expression in ascites cells (9, 10). In the present study, the TLR4 expression in cells of macrophage lineage was highly correlated with the TNF-α production of ascites cells and clinical responses to OK-432, suggesting that TLR4 expression in ascites cells may be a novel predictor in OK-432 immunotherapy for malignant ascites from gastric cancer.

References


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