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Profile of Cytokines Produced in Tumor Tissue after Administration of Cyclophosphamide in a Combination Therapy with Tumor Necrosis Factor

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Abstract. *Background: Recent studies have shown that locoregional administration of tumor necrosis factor (TNF) or production of endogenous TNF in tumor tissue with a chemotherapeutic agent showed considerable antitumor effect without severe toxicities. To analyze the immunological role of chemotherapeutic agents in locoregional TNF therapy, the effect of cyclophosphamide (CY) on cytokine production after ONO-4007 treatment was examined. Materials and Methods: Seven days after inoculation of Meth A fibrosarcoma in BALB/c mice, CY (100-150 mg/kg) was injected intraperitoneally and, after another 7 days, ONO-4007 (30mg/kg) was injected intravenously. A competitive PCR quantitative analysis of the induced cytokine messenger RNA (mRNA) was performed for IL-12 (p40), TGF-β, TNF-α, IL-1β, IL-10 and IL-6. Results: The antitumor effect of ONO-4007 was enhanced by preadministration of CY. CY significantly enhanced IL-12 (p40) mRNA compared to that by ONO-4007 alone. No increase in TGF-β mRNA was observed in either treatment. Conclusion: The preadministration of CY enhances IL-12 production in tumor tissues and shifts the cytokine profile to the dominant Th1 type.*

Systemic administration of cytokines to regress tumors causes serious side-effects such as severe fever and hypotension (1-3). Consequently, currently it is not possible to administer sufficient cytokines using a systemic route (4). However, recent studies have revealed that locoregional administration of TNF or enhancing the production of endogenous TNF in tumor tissue showed considerable antitumor effect without severe toxicity (5-9). In Europe, an isolated perfusion using TNF combined with melphalan, a chemotherapeutic agent, caused a drastic regression of unresectable melanoma and soft tissue sarcoma in the limbs (5, 6).

It has been reported that several weeks are necessary to cause an antitumor effect using an isolated TNF perfusion combined with a chemotherapeutic agent (10). This suggests that the antitumor effect of this therapy might be attributable to several indirect factors that are induced by the administration of TNF. Selective cytotoxicity against tumor microvessels by TNF is thought to be the main antitumor effect in this therapy (11). However, the indirect factors have not yet been elucidated. Analyses of the antitumor mechanisms of this therapy has been hampered because there is not a suitable animal model for isolation perfusion therapy. In this study, lesional TNF-inducing therapy combined with a chemotherapeutic agent was used as the model for isolated TNF perfusion. There is a phenotypic resemblance of antitumor effects from this model with isolated TNF perfusion; this can be demonstrated as follows (8, 12): (i) high concentrations of TNF can be specifically obtained around tumor tissues; (ii) to induce full antitumor effects, TNF and a chemotherapeutic agent are required; (iii) multiple focal necrotic tumor areas are observed after a single administration of the chemotherapeutic agent. These observation are unlike the single central necrosis that is observed after induction of endogenous TNF.

As we reported previously, ONO-4007 (a synthetic lipid A derivative) induces endogenous TNF specifically around tumor tissues; it has antitumor effects in the animal model without severe side-effects. However, a single administration is not enough to induce complete tumor regression (13). Cyclophosphamide (CY) was found to augment the

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Table I. *Sequence of PCR primers.*

Target gene		Primer Sequence (5'→3')	Size (bp)
IL-1β	S:	GGCTGCTTCCAAACCTTTGA	717
	A:	GAAGACACGGATTCCATGGT	
IL-6	S:	GCTATGAAGTTCCTCTCTGC	640
	A:	ACTAGGTTTGCCGAGTAGATC	
IL-10	S:	CTGGCATGAGGATCAGCAGG	386
	A:	CACCTGCTCCACTGCCTTGC	
IL-12p40	S:	ACTGGACCAAAGGGACTATG	431
	A:	AATAGCGATCCTGAGCTTGC	
TNF-α	S:	AGCACAGAAAGCATGATCCG	422
	A:	GGAGTAGACAAGGTACAACC	
TGF-β	S:	CCTGAGTGGCTGTCTTTTGA	579
	A:	GCGCACAATCATGTTGGACA	

Table II. *Time period between administration of CY and ONO-4007 and effectiveness of tumor regression in Meth A fibrosarcoma.*

CY (mg/kg)	ONO-4007 (mg/kg)	Interval 1day	4day	7day	10day
0	0	NT*	NT	0/6**	NT
0	30	NT	NT	0/5	NT
150	0	NT	NT	0/5	NT
100	30	NT	NT	4/5	NT
150	30	1/5	4/5	5/5	1/5

*: Not tested

**: Number of mice with complete tumor regression/Number of tested mice

Meth A cells ($2x10^5$ cells) were inoculated intradermally into the abdomen of BALB/c mice. CY (100 or 150mg/kg) was administered on day 7. ONO-4007 (30mg/kg) was injected intravenously on day 8, 11, 14, or 17.

antitumor effects of ONO-4007 in a mouse tumor model (13). Therefore, it appears that a useful combination therapy would utilize CY as the chemotherapeutic agent with ONO-4007 to induce locoregional TNF.

We hypothesize that CY may induce macrophages to produce cytokines, these cytokines then assist the TNF in regressing tumors. To test this hypothesis, the cytokine mRNAs that are induced around tumor lesions were measured quantitatively by reverse transcription polymerase chain reaction (RT-PCR). This data was used to evaluate whether premedication of CY causes a change in the profile of the cytokines that are produced after treatment by ONO-4007.

Materials and Methods

Mice. BALB/c and C3H/He male mice were purchased from Shizuoka Experimental Animal Farm (Shizuoka, Japan) or Seac Yoshitomi (Fukuoka, Japan). They were 6-12 weeks of age at the start of the experiments. All mice were given a standard laboratory diet and water *ad libitum*.

Tumors. Meth A fibrosarcoma and MH134 hepatoma were passaged once a week as ascites in BALB/c or C3H/He mice, respectively.

Reagents. CY was purchased from Wako Pure Chemical Industries (Osaka, Japan). ONO-4007, a synthetic lipid A derivative (14-16), was donated by Ono Pharmaceutical Company (Osaka, Japan).

Therapeutic experiment. Meth A fibrosarcoma (2×10^5 cells) was inoculated *i.d.* into the abdomen of BALB/c mice. CY (100-150 mg/kg) was administered intraperitoneally (*i.p.*) on day 7 and endogenous TNF induction was performed on day 14 after the tumor inoculation by *i.v.* administration of ONO-4007 (30mg/kg). The degree of tumor regression was estimated four weeks after tumor inoculation.

Induction of endogenous TNF in tumor lesion. Details of the procedure to obtain TNF-containing samples was described previously (17). Briefly, Meth A fibrosarcoma bearing mice (BALB/c) were treated with ONO-4007. Ninety minutes after injection, tumor tissues were taken, weighed, minced and homogenized. The supernatant of the homogenized sample obtained by centrifuge (7000g x 10min) was kept at -80˚C until use. TNF in the supernatant was assayed by the method previously described (17, 18).

Reverse transcription/polymerase chain reaction (RT-PCR). Meth A fibrosarcoma-bearing BALB/c mice were administered CY (150 mg/kg) on day 7 after tumor inoculation and treated with an endogenous TNF inducer (ONO-4007 30mg/kg) on day 14 after tumor inoculation. One to 24 hours after injection of ONO-4007, tumor tissues were taken; total RNA was extracted using an acid-guanidium-phenol-chloroform method. Expressions of mRNA of the cytokines were detected by RT-PCR. Briefly, PCR was performed for 40 cycles, each consisting of denaturation for 1 minute at 94˚C, annealing for 2 minutes at 60˚C and extension for 1 minute at 72˚C. The PCR products were electrophoresed on agarose gel containing ethidium bromide. The sequence of PCR primers is shown in Table I. Competitive PCR was performed for the quantitative analysis of the induced cytokine mRNA. The competitive templates were made with a Competitive DNA Construction Kit (Takara, Japan) using sense and antisense primers, which can amplify the mRNAs for IL-12 p40, TGF-β, TNF-α, IL-1β, IL-10 and IL-6.

Statistical analysis. Statistical evaluations of differences between groups were made by Student's *t*-test and ANOVA with the Dunett test.

Results

Antitumor effects of the combination therapy with CY and ONO-4007. In this study, ONO-4007 (a synthetic lipid A derivative with low molecular weight) was used to induce endogenous TNF (14-16). The optimal time interval

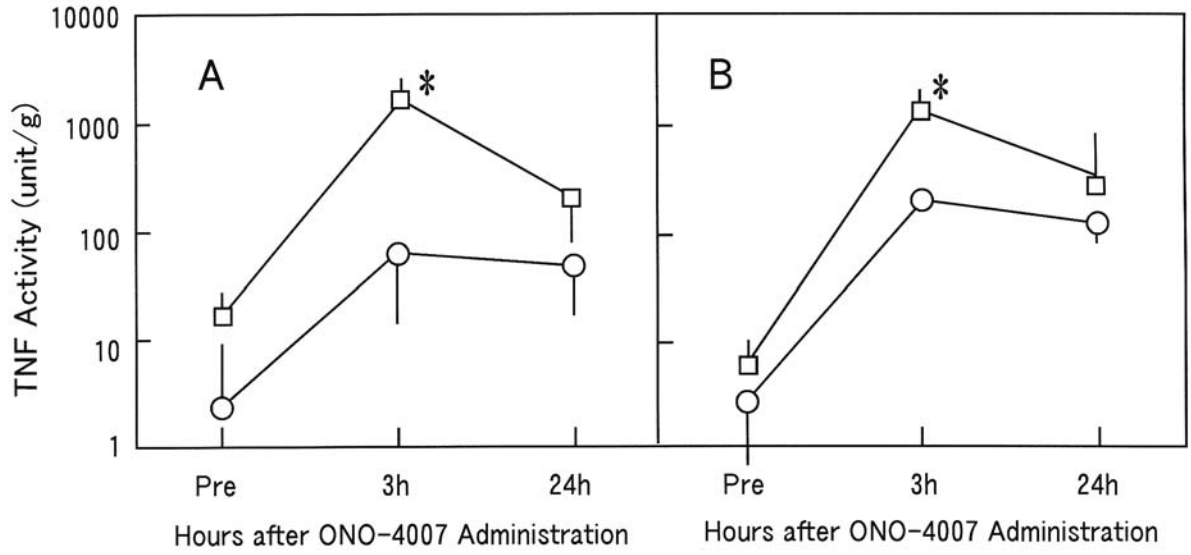

Figure 1. *Endogenous TNF activity in tumor tissue after ONO-4007 administration. MethA-bearing BALB/c mice (A) or MH134-bearing C3H/He mice (B) were treated with CY (150mg/kg) on day 7 and were intravenously administered ONO-4007 (30mg/kg) on day 14. Tumor tissue was collected 0, 3 and 24 hours after ONO-4007 administration. Tumor tissues were homogenized and centrifuged as described in Materials and Methods. (○), Saline+ONO-4007: (□), CY and ONO-4007. *: Significant differences from the saline+ONO-4007 control ($p<0.01$, Student's t-test).*

between CY and ONO-4007 administration was determined with the intradermally inoculated Meth A tumor model. It had been previously shown that the antitumor effect by endogenous TNF (induced by LPS (13), or by a combination of interferon-γ and OK-432 (12)) was enhanced by CY premedication 7 days before induction of endogenous TNF. As shown in Table II, the antitumor effect of ONO-4007 was also enhanced the most by CY premedication that occurred 7 days before ONO-4007 administration.

TNF production in tumor tissues after a combination treatment of both ONO-4007 and CY. One of the antitumor mechanisms of the combination therapy is the augmentation of TNF production in the tumor tissues. Therefore, we examined whether CY enhanced TNF production in the tumor tissues that had been treated with ONO-4007.

Intradermally inoculated Meth A in BALB/c mice or MH134 in C3H/He mice was used. CY and ONO-4007 were administered as described in Materials and Methods. Tumors were removed at 3 and 24 hours after ONO-4007 administration and the amount of TNF in the tumor was measured by bio-assay. The results are shown in Figure 1. In both Meth A and MH134 tumors, premedication with CY significantly enhanced the intratumor TNF activity that was induced by ONO-4007. This data indicates that CY can be effective in enhancing TNF production independently of tumor origin.

Quantification of mRNA of cytokines in tumor tissues after combination therapy with CY and ONO-4007. Competitive PCR was used to measure the amount of cytokines in tumor tissues after CY and ONO-4007 administration. Meth A fibrosarcoma bearing mice (BALB/c) were treated with CY and ONO-4007 as described in Materials and Methods. Tumors were removed at 1, 3, 6 and 24 hours after ONO-4007 administration. RNA was extracted from tumor specimens and the number of copies of mRNA of each cytokine was measured by competitive PCR methods. As shown in Figure 2, the number of copies of mRNA of the IL-12 p40 subunit, TNF-α, IL-1β, IL-6 and IL-10 was enhanced about 10-100 times 3 hours after administration of ONO-4007, and returned to the un-stimulated level after 24 hours. Administration of CY significantly enhanced the amount of IL-12 mRNA compared to that by ONO-4007 alone. In the case of TGF-β, no increase in mRNA was observed in either treatment. These results suggest that preadministration of CY enhances IL-12 production in the tumor tissues and it may lead to a shift in the cytokine profile to dominant Th1 type.

Discussion

Because the antitumor effect of ONO-4007 is inhibited by the administration of anti-TNF antibody (14), TNF is undoubtedly the main (effector) molecule that exerts antitumor effects during the combination therapy. This suggests that one of the antitumor mechanisms of CY is its

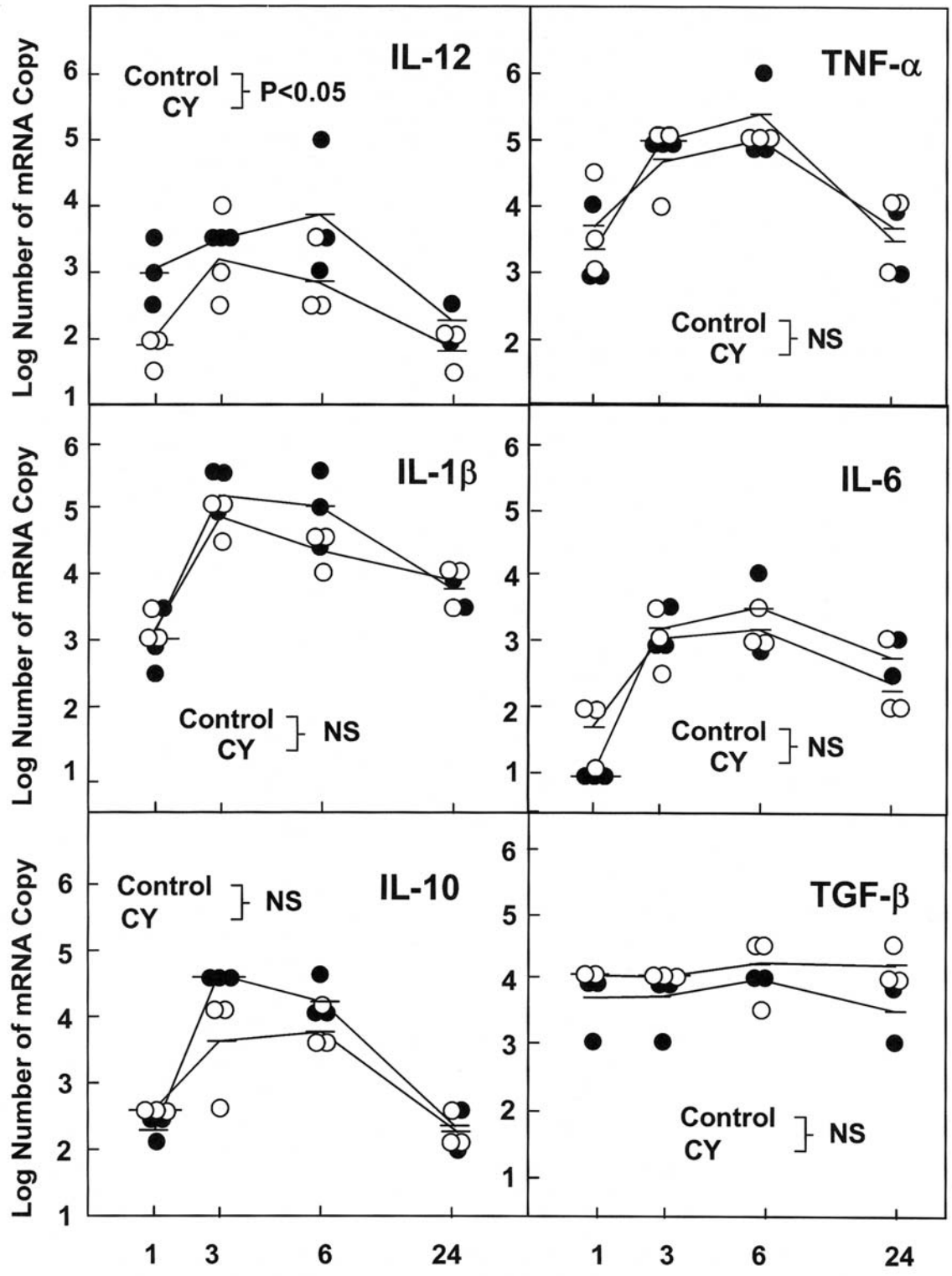

Figure 2. *Expression number of various cytokines mRNA in tumor tissues treated with CY and ONO-4007. Meth A fibrosarcoma-bearing mice were administered CY (150mg/kg) on day 7 and treated with ONO-4007 (30mg/kg) on day 14 after tumor inoculation. Tumor tissues were taken and expression numbers of mRNA of cytokines (IL-12, TNF-α, IL-1β, IL-6, IL-10 and TGF-β) were detected by RT-PCR as described in Materials and Methods. (○), Saline+ONO-4007: (●), CY and ONO-4007. Significant differences between the control (only ONO-4007) and CY (CY + ONO-4007) were analyzed by analysis of variance (ANOVA) and Dunnett two-tailed test.*

effect on the macrophages which are the main cells producing TNF in the tumor lesions. The mechanism by which CY enhances TNF production after treatment with ONO-4007 can be ascribed to the following: (i) there is an increase in the number of macrophages in the tumor lesion; and/or (ii) the macrophages are activated to produce a larger amount of TNF. The existence of tumor-associated macrophages (TAMs) in tumor tissues may play an essential role in regressing tumors (19). CY may cause an increase in the number of TAMs (20). Moreover, CY up-regulated Mac-1, Fc receptors and Class II antigens on the TAMs (20, 21). These results suggest that the administration of CY might enhance TNF production by TAMs through the mechanisms provoked by Mac-1 and FcR.

Figure 2 shows that CY significantly enhanced the production of IL-12 p40 subunits in tumor tissues. IL-12 consists of the heterodimer of the subunit of p35 and p40 (22); the p35 subunit is constitutively produced in macrophages and the p40 subunit is induced in activated macrophages. IL-12 is one of the key cytokines that cause a shift in the cytokine profile to the Th1 type (both NK cells and macrophages are activated and cytotoxic T lymphocytes are generated). These appear to be the principle mechanisms in regressing tumors especially *in vivo* (23). CY appears to have a role in regulating the cytokine profile in tumor lesions by shifting to the Th1 type.

The isolated TNF perfusion method appears to be a novel method for enhancing antitumor effects. Locoregional administration of TNF with a chemotherapeutic may be an effective new method for curing cancer. It has been reported that the TNF in an isolation perfusion selectively injures microvessels in tumor tissues (11) and enhances the permeability of endothelial cell membranes of microvessels in the tumor tissues; this results in increased delivery of the chemotherapeutic agent to tumor cells (10, 24). However, some antitumor effects occur several weeks after the treatment. Thus, various unknown mechanisms may be involved in some of the fairly strong antitumor effects that occur after locoregional treatment with a TNF and chemotherapeutic agent.

This study demonstrated, for the first time, that locoregional administration of TNF with CY can modulate the cytokine profile to the dominant Th1 type through the enhanced induction of IL-12 in tumor lesions. Further investigation will be needed to seek the best chemotherapeutic agent to be used in combination; this can be determined by monitoring cytokine production, especially IL-12.

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