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The Synergistic Antitumor Effect of Natural-Human TNF and Anticancer Drugs

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ABSTRACT

In the present report, we compared and discussed synergistic antitumor effects of natural-human tumor necrosis factor (n-TNF) which was derived from human acute lymphoblastic leukemia BALL-1 cells and conventional anticancer drugs by using Lewis lung carcinoma which was transplanted on BDF_1 mice. n-TNF and anticancer drugs were administered daily for 10 days. n-TNF showed antitumor effects which were equivalent to or stronger than MMC (1 mg/kg/day, i.v.), 5FU (5 mg/kg/day, i.v.), Adriamycin (1 mg/kg/day, i.v.), Actinomycin D (0.05 mg/kg/day, i.v.), Cyclophosphamide (10 mg/kg/day, i.v.) and OK-432 (0.5 KE/mouse/day, s.c.).

And synergistic antitumor effects were observed when n-TNF was administered with anticancer drugs, and the strong enforcement was obtained especially when it was combined with 5FU.

The antitumor effect of tumor necrosis factor (TNF) has been reported since the reports by Carswell et al[2] and Currie et al[3], and TNF is remarked as immuno-therapeutic agent against carcinomas.

Recently the identity of TNF and the macrophage-secreted factor cachectin was reported[1] and the side effect of TNF will be a problem in clinical use in spite of its strong antitumor effect. Therefore, we studied the combination therapy of TNF and conventional anticancer drugs for obtaining a strong antitumor effect without side effects. In this present study, we compared the antitumor effect of natural type human TNF (n-TNF)[8] and conventional anticancer drugs, and secondary discussed the synergistic antitumor effects of the combination dose of n-TNF and conventional anticancer drugs.

MATERIALS AND METHOD

n-TNF

Natural-human TNF was supplied by Hayashibara Biochemical Laboratories, Inco., Okayama, and used. n-TNF, with the molecular weight of 17,000, is a protein at the isoelectric point from 5.2 to 6.2 which is stable at 56°C for 30 min, and which has the 161 amino acids sequence[6]. Its cytotoxicity was determined by the high sensitive and rapid assay of Eifel et al[4] for lymphotoxin, using mice L929 cell as the target cell. One unit of activity is designated as the reciprocal of the dilution that effects cytopathic effect (CPE) in 50% of the target cells. In this series, n-TNF 1×10^4U/kg/day was administered daily for 10 days into the tail vein of mouse.

Anticancer drugs

Mitomycin C (MMC) (1 mg/kg/day), 5-Fluorouracil (5FU) (5 mg/kg/day), Adriamycin (ADM) (1 mg/kg/day), Actinomycin D (ACM-D) (0.05 mg/kg/day), Cyclophosphamide (Cyclo.) (10 mg/kg/day) and OK-432 (0.5 KE/mouse/day) were commercially available and used. The five former drugs were administered intravenously into the tail vein and the latter was administered subcutaneously into the back of mouse daily for 10 days.

Experimental animal

Eight weeks old female BD (C57BL/6 × DBA/2) F_1 mice, about 25 g each, were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan, and used in this series.

Tumor

Lewis lung carcinoma (3LL) cells successively maintained subcutaneously in C57BL/6 mice by the First Department of Surgery, Okayama University Medical School were used. For the experiment, the tumor was excised aseptically on the 10th day from the successive transplantation, minced, washed three times in Hanks solution, treated with 0.25% trypsin, Difco Co., USA, at 37 C for 15 min, washed twice with Eagle's essential medium supplemented with 10% FCS, filtered through #80 and #150 wire meshes and prepared into single cells.

Experimental design of mice pulmonary metastatic tumors

BDF_1 mice were inoculated to their left foot pad 1×10^6 3LL cells, and these tumors were regarded as the primary tumor. The primary tumor was removed by femoral amputation under ether anesthesia on th 10th day from the inoculation. After the amputation, mice were mixed up and divided into each group. The administration was started on the next day from the amputation of the primary tumor and continued for 10 days. In the combine dose group, n-TNF and anticancer drugs were injected, separately. The mice pulmonary metastatic tumors were evaluated on the 21th day from the inoculation. The evaluation was carried out by the Wexler's method[12] (Fig. 1).

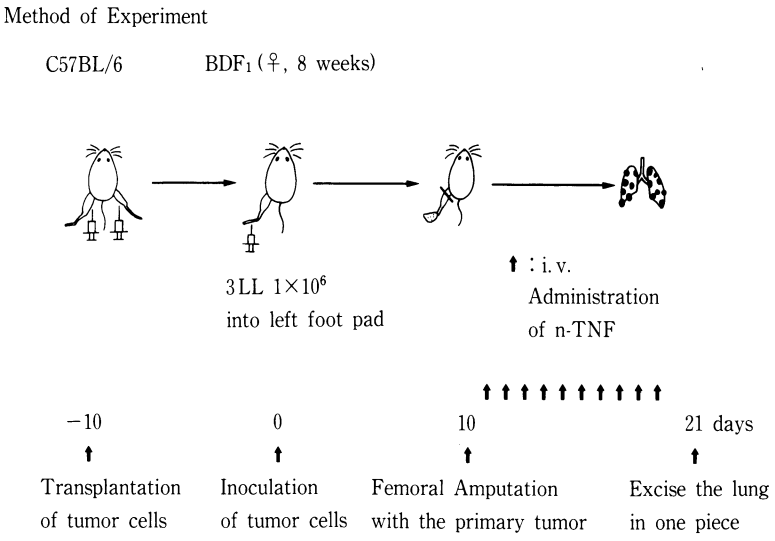

Fig. 1. Experimental design of mice pulmonary metastatic tumors

Study of the mice body weight and splenic weight

Mice body weight was measured on the 21th day from the inoculation, just before mice were sacrificed. And spleens were resected after mice were sacrificed, and weight was measured.

Statistical analysis was carried out by Student's t-test.

RESULTS

Antitumor effects of the single dose of n-TNF and each of anticancer drugs

Table 1 shows results of antitumor effects of single dose groups and combine dose groups. n-TNF showed the inhibition rate of the tumor proliferation of 71.2% ($p<0.01$) on the single dose. MMC showed 46.9% ($p<0.05$), 5FU showed 7.4% ($p<0.01$), ADM showed 61.1% ($p<0.01$), ACM-D showed 21.9% (not significant), Cyclo. showed 70.9% ($p<0.01$) and OK-432 showed 52.9% (not significant).

n-TNF showed the effect equivalent to those of 5FU, ADM and Cyclo., and it was more effective than MMC, ACM-D and OK-432 on the dose in the present study.

Table 1. Antitumor effects of single dose groups and combine dose groups of n-TNF and conventional antitumor drugs

		n	Pulmonary tumor inhibition rate(%)[a]	Pulmonary Tumor: Range of No. of pulmonary tumor (mean ± SD)	Incidence	P(t-test)
Saline	(0.2ml)	7	0	19~65(43.7 ± 16.5)	7/7	—
n-TNF	(1×10^4U/kg/day)	7	71.2	4~35(12.6 ± 10.6)	7/7	0.01
MMC	(1mg/kg/day)	6	46.9	3~43(23.2 ± 15.6)	6/6	0.05
5FU	(5mg/kg/day)	7	57.4	5~37(18.6 ± 12.5)	7/7	0.01[b]
ADM	(1mg/kg/day)	7	61.1	6~35(17.0 ± 11.1)	7/7	0.01
ACM-D	(0.05mg/kg/day)	7	29.1	2~46(31.0 ± 15.1)	7/7	not significant
Cyclo.	(10mg/kg/day)	5	70.9	3~20(11.4 ± 6.3)	5/5	0.01
OK-432	(0.5KE/day)	5	52.9	6~45(20.6 ± 16.0)	5/5	not significant
MMC	+n-TNF	7	70.3	0~35(13.0 ± 13.0)	5/7	0.01
5FU	+n-TNF	7	84.7	0~17(6.7 ± 6.5)	5/7	0.001[c]
ADM	+n-TNF	7	59.5	3~32(17.7 ± 12.1)	7/7	0.01
ACM-D	+n-TNF	7	43.7	9~48(24.6 ± 15.7)	7/7	0.05
Cyclo.	+n-TNF	5	62.5	2~32(16.4 ± 12.9)	5/5	0.01
OK-432	+n-TNF	4	42.8	7~39(25.0 ± 14.1)	4/4	not significant

ADM; Adriamycin, ACM-D; Actinomycin D, Cyclo.; Cyclophosphamide

a) pulmonary tumor inhibition rate

$$= \left(1 - \frac{\text{mean numbers of pulmonary metastatic tumors in each group}}{\text{mean numbers of pulmonary metastatic tumors in each group}} \right) \times 100\%$$

b) vs c) : $p<0.05$

Antitumor effects of the combine dose of n-TNF and anticancer drugs

When n-TNF was administered with other anticancer drugs, MMC puls n-TNF showed the inhibition rate of the tumor proliferation of 70.3% ($p<0.01$), 5FU puls n-TNF showed 84.7% ($p<0.001$), ADM puls n-TNF showed 59.5% ($p<0.01$), ACM-D puls n-TNF showed 43.7% ($p<0.05$), Cyclo. puls n-TNF showed 62.5% ($p<0.01$) and OK-432 puls n-TNF showed 42.8% (not significant). When each anticancer drugof 5FU, MMC or ACM-D and n-TNF were combined, stronger antitumor effects than the single dose of each anticancer drug were obtained. And no metastatic tumors were observed in 2/7 cases for combine dose groups of 5FU puls n-TNF and MMC puls n-TNF, respectively. In addition, the statisticallly significant enforcement of antitumor effects was obtained for the combinedose group of 5FU puls n-TNF ($p<0.05$) in comparison with the signle dose group of 5FU.

Mouse body weight and splenic weight after the single or combine dose of n-TNF and each of anticancer drugs

There were no changes in body weight and splenic weight after the single dose of n-TNF. But the single doses of MMC and Cyclo. brought about statistically significant decreses in body weight. Also, single dose groups of MMC, ADM, ACM-D, Cyclo. showed statistically significant decreases in splenic weight. Among combine dose groups of n-TNF with each of anticancer drugs, those which showed decreases in body weight or splenic weight were decreased in number in comparison with single dose groups; only, decreases in body weight for the Cyclo. puls n-TNF groupand decreases in splenic weight for the MMC puls n-TNF group were observed (Table 2). Provided that decreases in body weight and splenic weight are side effects by drugs, we

Table 2. Mice body weight and splenic weight in single dose groups and combine dose groups

		n	Body weight(g)[a] (mean ± SD)	P(t-test)	Splenic weight(g)[b] (mean ± SD)	P(t-test)
Saline	(0.2ml)	7	20.9 ± 2.2	—	0.14 ± 0.03	—
n-TNF	(1×10^4U/kg/day)	7	19.7 ± 1.7		0.17 ± 0.03	
MMC	(1mg/kg/day)	6	18.9 ± 1.7	0.1	0.06 ± 0.02	0.01
5FU	(5mg/kg/day)	7	20.6 ± 1.3		0.15 ± 0.04	
ADM	(1mg/kg/day)	7	19.4 ± 1.5		0.09 ± 0.03	0.01
ACM-D	(0.05mg/kg/day)	7	20.4 ± 1.0		0.08 ± 0.04	0.01
Cyclo.	(10mg/kg/day)	5	17.6 ± 4.3	0.05	0.14 ± 0.03	0.1
OK-432	(0.5KE/day)	5	18.8 ± 1.9		0.20 ± 0.02	
MMC	+n-TNF	7	20.4 ± 1.7		0.04 ± 0.01	0.01
5FU	+n-TNF	7	20.1 ± 1.8		0.15 ± 0.03	
ADM	+n-TNF	7	20.6 ± 1.7		0.08 ± 0.01	
ACM-D	+n-TNF	7	19.1 ± 1.3		0.12 ± 0.01	
Cyclo.	+n-TNF	5	16.8 ± 1.9	0.01	0.15 ± 0.04	
OK-432	+n-TNF	4	18.3 ± 2.1		0.22 ± 0.03	

ADM; Adriamycin, ACM-D; Actinomycin D, Cyclo.; Cyclophosphamide

a) Body weights were measured just before the sacrifice on the 21th day from the inoculation.
b) Splenic weights were measured after sacrifice on the 21th day from the inoculation.

can say that the safety of the single dose and combine dose of n-TNF was suggested.

DISCUSSION

Many investigations have been continued since Carswell et al[2] and Currie et al[3] reported on TNF. Until a recent date, obtaining a large amount of purified TNF was difficult. However, it became possible for us to obtain human TNF of a large amount and also of natural type by the "Hamster Method"[6]. Discussing antitumor effects of this natural-human TNF, we have already clarified the following effects[8]; (1) It has antitumor effects dose dependently. (2) The continuous administration is effective. (3) Each route of the administration of i.v., i.m. and i.t. is effective. (4) It is suggested that n-TNF effects tumor cells directly and in association with the cell cycle. And the safety of n-TNF dose was suggested because decreases in mice body weight and splenic weight were not observed after the administration of n-TNF.

On the other hand, anticancer drugs which are already in practical use have not only antitumor effects but also some side effects, which have given rise to a problem in clinical use. It is already known that the combination chemotherapy is being done for the purpose of enforcing antitumor effects and decreasing side effects, by the combine dose of each of anticancer drugs. Recently, the combination therapy of conventional anticancer drugs with lymphokine, especially with lymphotoxin[9] or interferon[13] is also being tried.

In the present study, we compared antitumor effects of the single dose of natural-human TNF and five anticancer drugs and also OK-432 which was an immuno-activator, and also discussed synergistic antitumor effects on the combine dose. n-TNF showed antitumor effects which were equivalent to 5FU, ADM and Cyclo. on the single dose. And n-TNF showed stronger antitumor effects than MMC, ACM-D and OK-432 in the dose concentration in our experiments. Enforced antitumor effects were obtained in the combine dose with MMC, 5FU and ACM-D respectively than in the single dose of each. Especially as to 5FU, statistically significant enforcement of antitumor effects was observed on the combine dose with n-TNF compared with on the single dose. 5FU is an antimetabolite and a drug which inhibits DNA synthesis of tumor cells and gives its antitumor effects[5]. On the other hand, it is shown in experiments by Naomoto et al[10] that n-TNF, which was discussed in the present study, accumulates tumor cells on the S phase of the cell cycle. The appearance of strong synergistic antitumor effects of the combine dose of 5FU and n-TNF which was shown in the present study will be explained, from the above viewpoints of the cell cycle. Since MMC, ADM, ADM-D and Cyclo. are also

drugs which act relating with synthesis of DNA or RNA in tumor cells, further enforcement of synergistic effects of the combine dose can be expected by discussing the dosage and the timing of the administration. Significant antitumor effects were not observed on the combine dose with OK-432[7] which is an immunoactivator in the present study. As OK-432 is known to induce the interferon gamma[11] the timing the combine administration with n-TNF and the study of dose are tasks for the future.

Changes in mice body weight and splenic weight after the administration of n-TNF, anticancer drugs or OK-432 were considered to be a factor of side effects, and discussed. n-TNF, 5FU or OK-432 did not change either body weight or splenic weight, while 4 anticancer drugs, that is, MMC, ADM, ACM-D and Cyclo. decreased both or either of body weight and splenic weight statistically significantly. When these 4 anticancer drugs were combined with n-TNF, body weight loss was observed only in the Cyclo. puls n-TNF group and splenic weight loss was observed only in the MMC puls n-TNF group, though the same amount as in the single dose was administered. The mechanism of this decreased side effect was unknown, but it fits to the purpose of the side effect decrease which is one of the purpose of the combination therapy.

From the facts that synergistic antitumor effects were observed and side effects were reduced on the combine dose of n-TNF and anticancer drugs as described above, natural-human TNF may well be used as a new antitumor agent and its future development is expected.

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