

Acta Medica Okayama

Volume 44, Issue 4

1990

Article 6

AUGUST 1990

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Abstract

To prevent the development of hepatic metastases after surgery for colorectal cancer, it is important to inhibit the growth of any micrometastases which occur during the operation. We used a hepatic metastasis model in mice to investigate the effects of combination therapy with natural human tumor necrosis factor- α (nHuTNF- α) and natural murine interferon- α/β (nMuIFN- α/β). Decreased formation of hepatic metastases by murine colon-26 carcinoma was recognized following a single injection of nHuTNF- α , nMuIFN- α/β , or both. These inhibitory effects were synergistic. NK activity was also measured, because natural killer cells not only have an anti-tumor effect but are also a representative of the host immune system. Both nHuTNF- α and nMuIFN- α/β were able to activate NK cells, and the combination of the cytokines more significantly augmented NK activity. The in vivo elevation of NK activity induced by nHuTNF- α , nMuIFN- α/β , or their combination may be one of the mechanisms of their antiproliferative effect on experimental hepatic metastases of murine colon-26 carcinoma.

KEYWORDS: nHuTNF- α , nMuIFN- α/β , antiproliferative effect, hepatic metastasis, NK activity

*PMID: 2244476 [PubMed - indexed for MEDLINE]

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To prevent the development of hepatic metastases after surgery for colorectal cancer, it is important to inhibit the growth of any micrometastases which occur during the operation. We used a hepatic metastasis model in mice to investigate the effects of combination therapy with natural human tumor necrosis factor- α (nHuTNF- α) and natural murine interferon- α/β (nMuIFN- α/β). Decreased formation of hepatic metastases by murine colon-26 carcinoma was recognized following a single injection of nHuTNF- α , nMuIFN- α/β , or both. These inhibitory effects were synergistic. NK activity was also measured, because natural killer cells not only have an anti-tumor effect but are also a representative of the host immune system. Both nHuTNF- α and nMuIFN- α/β were able to activate NK cells, and the combination of the cytokines more significantly augmented NK activity. The *in vivo* elevation of NK activity induced by nHuTNF- α , nMuIFN- α/β , or their combination may be one of the mechanisms of their antiproliferative effect on experimental hepatic metastases of murine colon-26 carcinoma.

Key words: nHuTNF- α , nMuIFN- α/β , antiproliferative effect, hepatic metastasis, NK activity

The inhibition of hepatic metastasis is an important prerequisite to decreasing the mortality rate of patients with colorectal cancer. Anticancer drugs are not usually effective against gastrointestinal tract cancers when they are administered generally. For this reason we investigated cytokine therapy using tumor necrosis factor (TNF) and interferon (IFN). TNF is a cytokine that was first recognized by Carswell *et al.* (1) by its lysis of tumor cells, and it is now known to have a variety of immunomodulatory functions in the host immune defenses (2-7). It exerts a strong

antitumor effect against some types of tumors both *in vivo* and *in vitro*. IFN is a group of cytokines that were originally detected by their ability to mediate antiviral activity. Their multiple biological activities, including *in vitro* and *in vivo* anti-tumor activity, are well known (8). Recent studies have indicated that the simultaneous use of TNF- α and IFN- α synergistically enhanced antitumor activity (9-12). This study investigated whether the combination of TNF with IFN could inhibit the growth of hepatic micrometastases in an experimental hepatic metastasis model in mice to provide a rationale for the development of clinical trials.

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Materials and Methods

Animals. Specific pathogen-free male CDF1 (BALB/c × DBA/2) mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), and were used at 6 weeks of age. Twenty-four mice were divided into four groups of six mice each.

Tumor. The tumor used in this study was mouse colon-26 undifferentiated colorectal adenocarcinoma, which was generously provided by Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). Cells were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS). The tumor cells were adjusted to a concentration of 1×10^4 cells/ml in phosphate-buffered saline (PBS) before use, and their viability was over 95%. The YAC-1 murine lymphoma cell line was purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Cells were maintained in RPMI 1640 medium supplemented with 10% FCS.

Reagents. Natural human TNF- α (nHuTNF- α) was derived from human acute B lymphoblastic leukemia cells (BALL-1 cells) stimulated by hemagglutinating virus of Japan (13), and its specific activity was 7×10^8 U/mg protein. Natural murine IFN- α/β (nMuIFN- α/β) was obtained from mouse L 202 cells, a cloned cell line of L 929 cells (14), and its specific activity was 1×10^7 IU/mg protein. These cytokines were generously provided by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan.

Experimental hepatic metastasis model. On day 0, mice were laparotomized under ether anesthesia, and colon-26 tumor cells ($5 \times 10^3/0.5$ ml/mouse) were inoculated into the portal vein via the superior mesenteric vein using a 30-gauge needle. After a sterile absorbable cotton sponge was placed over the injection site, the needle was withdrawn, and 10 sec later the sponge was removed without bleeding. On day 21, the mice were sacrificed, the livers were removed, and the metastatic colonies on the liver surface were counted with the naked eyes. The mean and standard deviation (S.D.) of the number of colonies in each group were calculated. The following equation was used to determine the rate of inhibition of metastasis:

Inhibition rate (%) =

$$\left[1 - \frac{\text{Mean number of liver metastases in experimental group}}{\text{Mean number of liver metastases in control group}} \right] \times 100$$

Experimental schedule. Twenty-four mice inoculated with colon-26 tumor cells were randomized into four

groups of six mice each on day 11. In a control group, mice were given saline 0.1ml/day successively through the tail vein from day 11 to 20. nHuTNF- α , nMuIFN- α/β , or a mixture of both cytokines was given to mice in the second, third, and fourth groups in the same way as for the control group.

Effector cells. A single-cell suspension of splenocytes was prepared by gently mincing spleens in PBS, passing them through #150 wire mesh, and purifying by centrifugation at 1600rpm for 30min on Ficoll-Hypaque (Pharmacia) gradients. Cells were then washed and adjusted to 5×10^6 /ml in RPMI 1640 medium supplemented with 10% FCS.

Target cells. The YAC-1 murine lymphoma cell line was used as an NK cell-susceptible target.

Assay for NK activity. The ^{51}Cr release assay of NK activity was performed as follows: The YAC-1 target cells were labeled with $100 \mu\text{Ci}$ of $\text{Na}_2^{51}\text{CrO}_4$ for 60min at 37°C . The cells were washed 3 times and adjusted to 1×10^5 /ml in the culture medium. Subsequently, effector cells and target cells were put in 96-well U-bottomed microtiter plates in a total volume of 200 μl and incubated for 4h at 37°C . Spontaneous ^{51}Cr release was determined by incubating target cells without effectors. After harvesting, the radioactivity in 100 μl of the supernatant was measured in a gamma counter. Cytotoxicity was calculated using the following formula. Specific lysis (%) =

$$\frac{\text{Test release} - \text{spontaneous release}}{\text{Total label} - \text{spontaneous release}} \times 100$$

All tests were performed in triplicate and the means were calculated.

Statistical analysis and determination of synergism. Statistical analysis was done by Student's *t*-test. To determine the synergism between nHuTNF- α and nMuIFN- α/β , we applied the median effect plot analysis described by Nishiyama *et al.* (15). This analysis allows dose-effect relationships in enzymatic, cellular and whole animal systems to be analysed. If the combination index was < 1.0 , the interaction of the two reagents was judged to be synergistic.

Results

Antitumor effects of nHuTNF- α in combination with nMuIFN- α/β against colon-26 metastatic liver tumors. nHuTNF- α , nMuIFN- α/β , or both (nHuTNF- α : nMuIFN- α/β ; 1:1) were

administered intravenously from the 11th to the 20th day after tumor inoculation at doses ranging from 10^2 to 10^4 U/kg/day (or IU/kg/day). As shown in Table 1, six mice were used in each group, and none of them died due to toxic effects of the exogenous cytokines. The antiproliferative effect of both nHuTNF- α and nMuIFN- α/β increased dose-dependently, and the combination of these cytokines had a greater antitumor effect than either cytokine alone at any concentration. This combination produced a greater than 50% reduction of hepatic metastases even at a dose of 10^2 U/kg/day (or IU/kg/day) when compared with the saline control group, and the difference was significant ($p < 0.02$). Thus, the antiproliferative effect of nHuTNF- α was augmented by addition of nMuIFN- α/β .

Combination index (CI) between nHuTNF- α and nMuIFN- α/β . Median effect plots were produced from the data shown in Table 1. A graphical presentation of CIs with respect to the fraction of cells affected (Fa) by nHuTNF- α and nMuIFN- α/β is shown in Fig. 1. The CI was

calculated to be 0.121 (< 1.0), indicating that the effect of the combination was synergistic.

In vivo activation of NK cells by nHuTNF- α and nMuIFN- α/β . On day 0, colon-26 tumor cells (5×10^3) were inoculated into the portal vein of CDF1 mice via the superior mesenteric vein. Injection of 0.1 ml of saline alone, nHuTNF- α , nMuIFN- α/β , or the combination of both cytokines started on the 11th day after inoculation, when no metastatic tumor colonies were seen yet on the liver surface with the naked eye. One group was treated with test materials only for 3 days, while the other group was treated for 10 days. Concentrations of nHuTNF- α and nMuIFN- α/β were 1×10^5 U/ml and 1×10^5 IU/ml, respectively. The cytotoxic effect of nHuTNF- α or nMuIFN- α/β alone was only modest, but when nHuTNF- α and nMuIFN- α/β were combined, their killing effect on YAC-1 cells was significantly enhanced after even 3 days of treatment, as well as after 10 days of treatment (Fig. 2).

Table 1 Antitumor effects of nHuTNF- α in combination with nMuIFN- α/β against colon-26 hepatic metastases^a

Reagents	Dosage ^b (UorIU/kg/day)	Number of mice	Number of metastases ^c	Inhibition rate (%) ^d	P values ^e
Saline		6	12.3 \pm 3.1	—	—
nHuTNF- α	1×10^2	6	9.0 \pm 2.8	26.8	N.S. ^f
nMuIFN- α/β	1×10^2	6	8.2 \pm 2.6	33.3	<0.05
nMuIFN- α/β +nHuTNF- α	1×10^2	6	5.7 \pm 4.3	53.7	<0.02
nHuTNF- α	1×10^3	6	8.0 \pm 3.0	35.0	<0.05
nMuIFN- α/β	1×10^3	6	6.7 \pm 1.4	45.5	<0.01
nMuIFN- α/β +nHuTNF- α	1×10^3	6	5.0 \pm 1.7	59.3	<0.001
nHuTNF- α	1×10^4	6	3.2 \pm 2.6	74.0	<0.001
nMuIFN- α/β	1×10^4	6	4.0 \pm 1.1	67.5	<0.001
nMuIFN- α/β +nHuTNF- α	1×10^4	6	2.8 \pm 1.8	77.2	<0.001

a: Colon-26 tumor cells were inoculated into the superior mesenteric vein of CDF1 mice on day 0. On day 21, the mice were sacrificed and the metastatic foci on the liver surface were counted.

b: Drugs were administered intravenously singly or in combination at the indicated doses from the 11th to the 20th day.

c: Mean value \pm standard deviation.

d: Calculated by the equation described in Materials and Methods.

e: Compared with the saline control group.

f: N.S.: not significant.

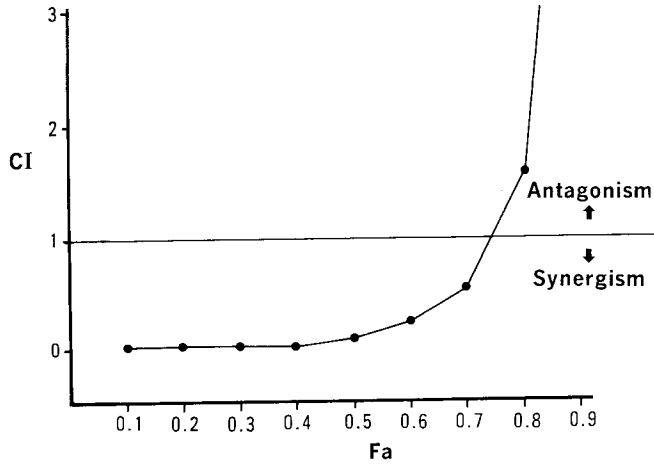


Fig. 1 The combination index (CI) with respect to the fraction affected (Fa) by synergistic inhibition with nHuTNF- α and nMuIFN- α/β .

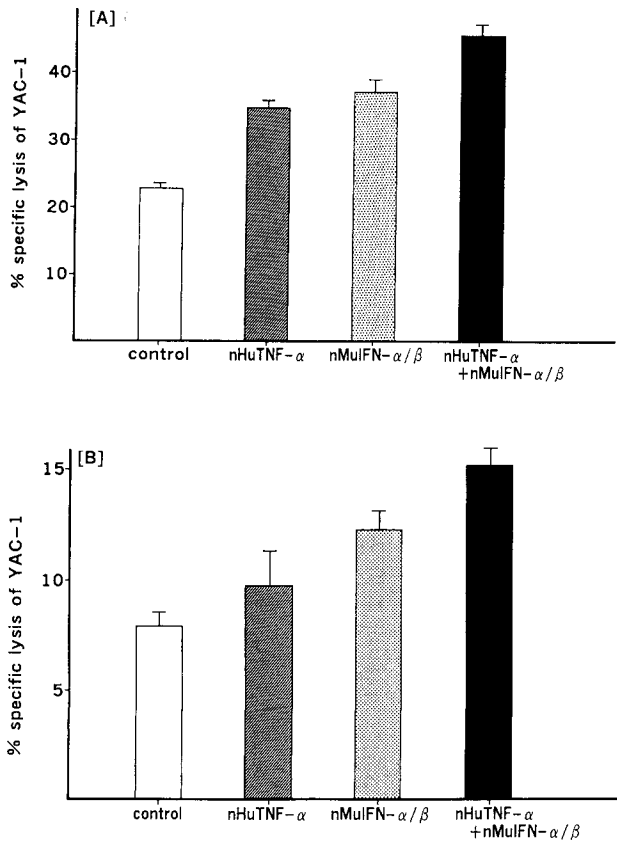


Fig. 2 Augmentation of cytolytic activity by nHuTNF- α and nMuIFN- α/β . On day 14(A) and day 21(B), mice were killed and the spleens were removed. NK activity in the spleen was then assessed at an effector/target ratio of 100:1

Discussion

In previous models of experimental hepatic metastasis, a variety of tumor cell lines and various routes of tumor injection have been used (16–19). However, most of them were unsuitable for investigating the efficacy of cytokines, which have indirect antitumor effects. Accordingly, we used the hepatic metastasis model of Tominaga *et al.* (20), in which colon-26 murine adenocarcinoma cells are injected into the superior mesenteric vein. This model renders the formation of metastatic nodules reliable.

Studies on the synergistic antitumor effects of TNF- α and IFN- α have been reported (9–12). Williamson *et al.* (9) and Orita *et al.* (10) independently reported the synergistic cytotoxic effect in *in vitro* studies, and Naomoto *et al.* analysed the synergistic effect by flow cytometry (11). Itano *et al.* reported a synergistic antitumor effect using a mouse lung metastatic model (12). However, there are few reports available on the synergistic antitumor effect of these cytokines in the experimental hepatic metastasis model.

Before our present experiment, the direct cytotoxicity of both nHuTNF- α and nMuIFN- α/β against colon-26 tumor cells was measured, and neither cytokine showed any direct cytotoxicity (data not shown). This result indicated that the cytotoxicity was an indirect effect mediated by host-immune effector cells. In fact, Hanna *et al.* reported that NK cells inhibited experimental tumor metastasis *in vivo* (21). We also tested the effects of these cytokines on NK activity, and detected that both nHuTNF- α and nMuIFN- α/β were able to significantly activate NK cells on day 14 or 21. Thus, the *in vivo* maintenance of elevated NK activity induced by nHuTNF- α , nMuIFN- α/β , or this combination may be one of the mechanisms of their antiproliferative effect against mouse colon-26 experimental hepatic metastases.

The results in Table 1 and Fig. 1 indicate that the antiproliferative effect of nHuTNF- α in com-

bination with nMuIFN- α/β was synergistic. These cytotoxic effects may be due to the enhancement of NK activity by these cytokines. Thus, combination therapy using these cytokines may be useful against hepatic metastases arising from human colonic cancer.

Acknowledgments. We would like to thank Dr. Nishiyama for his encouragement and advice during the present study. We also thank Mrs. Nasu for her help with the cell preparations.

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Received April 25, 1990; accepted June 6, 1990.