Preventive and Antiproliferative Effects of Tumor Necrosis Factor against Experimental Hepatic Metastases of Mouse Colon-26 Tumor

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This investigation was undertaken in order to assess both the preventive and antiproliferative effects of tumor necrosis factor (TNF) in a hepatic metastasis model, by means of inoculation of mouse colon-26 tumor cells into the portal vein via the superior mesenteric vein in male CDF1 mice, aged 5 weeks. Continuous 10-day administration of natural human TNF- α (nHuTNF- α) following the tumor cell inoculation caused no reduction but rather an increase in the number of hepatic metastases. However, pretreatment with this preparation daily for 10 days before the inoculation caused a remarkable decrease in the number of hepatic metastases. This prophylactic effect was reversed by the intravenous administration of anti-asialo GM1 antibody 24 h before the inoculation. The result of immunoperoxidase staining of liver specimens suggested that organ-associated natural killer cells might play a role in the metastatic inhibition. An apparent antiproliferative effect on metastatic liver tumors was also recognized following injection of nHuTNF- α from the 10th day after the inoculation. Thus, TNF appears to have important effects upon the host immune system, acting against liver metastases.

Key words: Tumor necrosis factor — Experimental hepatic metastases — Mouse colon-26 tumor — Preventive effect — Antiproliferative effect

The mortality rate owing to colorectal cancers is increasing year by year. Some of the possible reasons are as follows. At operation, solitary or multiple hepatic metastases are often found and postoperative hepatic recurrence is frequent in the advanced stages of colorectal cancer, even if the primary focus is completely removed. The high frequency of hepatic metastases is thus a crucial factor. Another is that anticancer drugs are usually not clinically effective against gastrointestinal tract cancers.

Tumor necrosis factor (TNF) is an antitumor cytokine derived from monocytes and macrophages and is known to exert a strong antitumor effect against some tumor cells both *in vitro* and *in vivo*. TNF also demonstrates many other biological effects; for example, it increases prostaglandin E₂ and collagenase production by human synovial cells and dermal fibroblasts, induces a procoagulant activity in vascular endothelial cells, and mediates the cytostatic properties of natural killer (NK) cells and macrophages. Therefore, we examined whether pretreatment with TNF could prevent mouse colon-26 cells from metastasizing into the liver and whether TNF could inhibit the growth of hepatic micrometastases in the experimental hepatic metastasis model which Tominaga *et al.* described.¹⁾

MATERIALS AND METHODS

Animals Specific pathogen-free male CDF1 (BALB/c $\times$ DBA/2) mice, aged 5 weeks, were purchased from Shizuoka Laboratory Animal Center, Shizuoka. They were housed under conventional environmental conditions and fed with normal mouse pellets and tap water *ad libitum*.

Tumor cells The mouse colon-26 tumor cells used here were generously provided by Asahi Chemical Industry Co., Ltd., Tokyo and had been derived from colorectal adenocarcinoma, which was induced as previously reported.²⁾ The tumor cells were serially subcultured *in vitro* every 5 days in RPMI 1640 medium (Nissui) supplemented with 5% fetal calf serum, and 1×10^4 cells/ml, with a viability of over 95%, were prepared in phosphate-buffered saline (PBS) prior to use.

Reagents Natural human TNF- α (nHuTNF- α) was purified from human acute B lymphoblastic leukemia cells (BALL-1 cells) stimulated by HVJ and was kindly supplied by Hayashibara Biochemical Laboratories, Inc., Okayama.³⁾ Its specific activity was 7×10^8 U/mg protein (2×10^6 JRU). The cytotoxicity of this preparation was determined by means of the highly sensitive and rapid assay of Eifel *et al.* for lymphotoxin, using ACD-treated mouse L 929 cells as the target.⁴⁾ One unit of activity is designated as the reciprocal of the dilution that has a cytopathic effect (CPE) in 50% of the target cells.

Lyophilized anti-asialo GM1 antiserum (Wako Pure Chemical Industries, Ltd., Osaka) was reconstituted with

Abbreviations: nHuTNF- α , natural human tumor necrosis factor- α ; HVJ, hemagglutinating virus of Japan; JRU, Japan Reference Unit; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

saline prior to use. As reported previously, anti-asialo GM1 antibody can completely eliminate NK activity *in vitro* when diluted in the presence of guinea pig complement (1:10).^{5,6} For the *in vivo* experiments, 0.1 ml (200 μ g) of anti-asialo GM1 antibody was administered into the tail vein of mice 24 h before the tumor inoculation.⁶

Experimental hepatic metastasis model On day 0, mice under ether anesthesia underwent laparotomy by an upper median incision, the duodenal loop was exposed, and a 0.5 ml aliquot of colon-26 tumor cells (5×10^3 cells) was inoculated into the portal vein via the superior mesenteric vein, through a 30-gauge needle attached to a 1-ml syringe. A sterile absorbable cotton sponge was placed over the injection site as the needle was withdrawn to prevent bleeding and peritoneal spillage of tumor cells. On day 21, the mice were killed and weighed. The livers of the mice inoculated with tumor cells were extracted and weighed, and the number of metastatic colonies on the surface was counted with the naked eye.

Administration schedule and dosage of nHuTNF- α The doses of nHuTNF- α used in this study ranged between 1×10^4 U/kg/day (28.6 JRU) and 1×10^5 U/kg/day (286 JRU). nHuTNF- α at a dose of 1×10^4 U/kg/day caused about 50% growth inhibition of Lewis pulmonary metastatic tumors transplanted into BDF1 mice, as Itano *et al.* have already reported.⁷

In the first protocol, the administration of this preparation into the tail vein of CDF1 mice was commenced 6 h after awakening from anesthesia on day 0. In the second protocol, nHuTNF- α was given intravenously from day 10. In the third protocol, pretreatment with this cytokine was performed between day -9 and day 0. On day 0, the last injection was finished 8 h before the inoculation and no additional treatment was given.

Statistical analysis was carried out by the use of Student's *t* test.

Immunoperoxidase staining (ABC method) A modification of the method of Hsu *et al.* was used.⁸ Twenty-one days after colon-26 tumor cell inoculation, the livers were removed and frozen immediately. Non-fixed frozen sections were cut into 5 μ m thick slices, air-dried for 1 h, and treated with acetone for 5 min at 4°C. After washing three times in PBS (pH 7.4), the sections were incubated for 15 min with methanol containing 0.3% H₂O₂ in order to remove the endogenous peroxidase. After washing three times in PBS, a normal horse serum (ABC kit PK-4002, Vector Labs, Burlingame, Calif.) was applied at 1:200 dilution for 20 min. The sections were then incubated overnight (12 h) at 4°C with the primary antiserum (rabbit anti-asialo GM1 antibody) at 1:50 dilution. Treatment with the primary antiserum was followed by the application of biotin-labeled secondary antibody (ABC kit PK-4002, Vector) for 30 min at a 1:200 dilution. After washing with PBS, the ABC complex (ABC kit PK-4002, Vector) was applied for 60 min. Brown staining was produced by treatment with a diaminobenzidine (DAB) solution consisting of 50 mg of DAB in 100 ml of PBS (pH 7.4) and 0.01% hydrogen peroxide. The slides were counterstained with Meyer-hematoxylin.

RESULTS

Influence of treatment with nHuTNF- α immediately after tumor cell inoculation on hepatic metastases of mouse colon-26 tumor cells Table I shows the influence of nHuTNF- α on hepatic metastases by 10-day continuous administration or by one-shot injection on day 0. Treatment with nHuTNF- α at a dose of 10^4 U/kg/day

Table I. Influence of Treatment with nHuTNF- α ^{a)} Immediately after Inoculation on Hepatic Metastases of Mouse Colon-26 Tumor Cells^{b)}

Day of injection	Dosage (U/kg/day)	No. of mice	Body weight ^{a)} (g)	Liver weight ^{c)} (g)	No. of colonies ^{c)}
Saline day 0-9	—	5	25.0 $\pm$ 1.7	1.76 $\pm$ 0.10	6.4 $\pm$ 6.1
nHuTNF- α day 0-9	1×10^4	5	26.6 $\pm$ 2.1	2.71 $\pm$ 0.72 ^{d)}	19.6 $\pm$ 10.3
nHuTNF- α day 0	1×10^5	5	24.8 $\pm$ 1.0	2.02 $\pm$ 0.30	8.8 $\pm$ 2.0

a) Six hours after inoculation, the administration of nHuTNF- α into the murine caudal vein was commenced at doses according to the experimental protocol.

b) Colon-26 tumor cells (5×10^3 cells) were inoculated into the portal vein of CDF1 mice via the superior mesenteric vein on day 0. On the 21st day, the mice were killed and the livers were removed.

c) Mean value $\pm$ standard deviation.

d) Liver weight was significantly increased compared with the control ($P < 0.05$).

Table II. Antiproliferative Effect of nHuTNF- α^a against Hepatic Metastases of Mouse Colon-26 Tumor Cells^{b)}

Day of injection	Dosage (U/kg/day)	No. of mice	Body weight ^{c)} (g)	Liver weight ^{c)} (g)	No. of colonies ^{d)}
Saline day 10-19	—	5	26.0 $\pm$ 0.8	1.98 $\pm$ 0.16	10.6 $\pm$ 3.7
nHuTNF- α day 10-19	1 $\times$ 10 ⁴	5	25.0 $\pm$ 1.7	1.69 $\pm$ 0.20	4.0 $\pm$ 2.1 ^{d)}
nHuTNF- α day 10, 12, 14, 16, 18	2 $\times$ 10 ⁴	5	22.4 $\pm$ 3.2	1.65 $\pm$ 0.34	3.0 $\pm$ 2.7 ^{d)}
nHuTNF- α day 10	1 $\times$ 10 ⁵	5	26.0 $\pm$ 1.4	0.89 $\pm$ 0.29	6.6 $\pm$ 2.1

a) From day 10, when micrometastases were expected to have become established, nHuTNF- α in the doses indicated by the experimental protocol was given intravenously.

b) Colon-26 tumor cells (5×10^3 cells) were inoculated into the portal vein of CDF1 mice via the superior mesenteric vein on day 0. On the 21st day, the mice were sacrificed and the livers were removed.

c) Mean value $\pm$ standard deviation.

d) Number of colonies was significantly decreased compared with the control ($P < 0.01$).

Table III. Prevention of Hepatic Metastases of Mouse Colon-26 tumor Cell^{a)} by Pretreatment with nHuTNF- α^b and the Antagonistic Action of Anti-asialo GM1 Antibody^{c)} (N=7)

No. of groups	Reagents	Body weight ^{d)} (g)	Liver weight ^{d)} (g)	No. of colonies ^{d)}
1	Saline	25.6 $\pm$ 1.3	1.80 $\pm$ 0.12	7.7 $\pm$ 3.3
2	Anti-asialo GM1 antibody	26.4 $\pm$ 1.0	2.32 $\pm$ 0.47 ^{e)}	49.1 $\pm$ 29.6 ^{e)}
3	nHuTNF- α	25.7 $\pm$ 0.5	1.65 $\pm$ 0.06 ^{e)}	1.6 $\pm$ 1.4 ^{f)}
4	nHuTNF- α + anti-asialo GM1 antibody	25.7 $\pm$ 1.0	2.04 $\pm$ 0.34	11.3 $\pm$ 6.9

a) Colon-26 tumor cells (5×10^3 cells) were inoculated into the portal vein of CDF1 mice on day 0. On the 21st day, the mice were killed and the livers were removed.

b) nHuTNF- α was administered into the tail vein at a dose of 2×10^4 U/kg/day between day -9 and day 0.

c) Anti-asialo GM1 antibody (200 μ g) was given intravenously on day -1.

d) Mean value $\pm$ standard deviation.

e) Significant differences ($P < 0.05$) between groups 1, 2 and 3 in liver weight; between groups 1 and 2 in number of colonies.

f) Significant differences ($P < 0.01$) between groups 1 and 3 in number of colonies.

* $P < 0.05$.

between day 0 and day 9 caused no reduction but rather an increase to 19.6 in the mean number of metastases from the value of 6.4 of the controls. The mean liver weight also increased in the TNF-treated group and statistical significance was shown in comparison with the control

group ($P < 0.05$). No change in body weight suggestive of side effects of nHuTNF- α was observed. A single injection of nHuTNF- α on day 0 caused no significant difference in the number of metastatic tumors from that of the control group.

Fig. 1. Hepatic metastatic tumors in mice which had received 10-day pretreatment with nHuTNF- α and were killed on day 21 after colon-26 tumor cell inoculation. Staining was done as described in "Materials and Methods"; Fig. 1A, Meyer-hematoxylin staining; Fig. 1B, anti-asialo GM1 antiserum staining. At 80 times magnification, marked lymphocytic infiltration around the tumor nodules can be seen, and most are asialo GM1-positive. Bar = 38 μ m.

Antiproliferative effect of nHuTNF- α against intra-hepatic micrometastases of mouse colon-26 tumor cell

In our preliminary experiments, metastatic tumors of colon-26 tumor could not be seen on the liver surface with the naked eye, but were found in the liver under a microscope on day 10. Therefore, we examined whether daily administration of nHuTNF- α from day 10 could inhibit the tumor growth in the liver. As shown in Table II, administration daily or every two days of nHuTNF- α reduced the mean number of colonies to 4.0 or 3.0, from the value of 10.6 of the controls, which was a significant growth inhibition ($P < 0.01$). However, a single injection of nHuTNF- α on day 10 did not significantly reduce the number of colonies.

Prevention of hepatic metastases of mouse colon-26 tumor cell by pretreatment with nHuTNF- α and the antagonistic action of anti-asialo GM1 antibody As hepatic metastasis inhibition was not observed on treatment with nHuTNF- α from day 0, the next experiment

was undertaken to assess metastasis prevention by pretreatment with this preparation. As shown in Table III, pretreatment with nHuTNF- α between day -9 and day 0 caused a considerable reduction in the number of metastatic colonies (1.6 ± 1.4). Treatment with anti-asialo GM1 antibody 24 h before the tumor inoculation increased the number of metastatic colonies (49.1 ± 29.6). The preventive effect of pretreatment with nHuTNF- α was abrogated by anti-asialo GM1 antibody. The same tendency was observed with regard to the mean liver weight. No variation in murine body weight was recognized.

Immunohistological examination Figure 1 shows hepatic tumors in a mouse which received 10-day administration of nHuTNF- α at a dose of 2×10^4 U/kg/day before the inoculation (Fig. 1A, Meyer-hematoxylin; Fig. 1B, anti-asialo GM1 antiserum). Figure 1A demonstrates marked lymphocyte infiltration around undifferentiated tumor cells, and in Fig. 1B, most of those lymphocytes are

stained positive. In contrast, few lymphocytes were found around the tumors and they stained negative with anti-asialo GM1 antiserum in the control group and in the group where nHuTNF- α was injected from day 10 to day 19 (data not shown).

DISCUSSION

Drugs and administration schedules to effectively prevent liver metastases of colorectal cancer have not yet been found. Further, a suitable experimental model of such liver metastasis for evaluating the efficacy of candidate drugs has not yet been established.

Some reports have appeared on *in vivo* experimental hepatic metastasis models produced by the inoculation of 3-methylcholanthrene-induced tumors into the spleen,⁹⁾ by inoculating Lewis lung carcinoma into the spleen,¹⁰⁾ and by other methods.^{11,12)} However, some of these cell lines were not derived from colorectal tumors of mice, and the metastatic tumors were impossible to count because of the poor reproducibility and the formation of infiltrative metastases. Naomoto *et al.* produced an experimental hepatic metastasis model of human colon adenocarcinoma cells, RPMI 4788 cells, transplanted into nude mice.¹³⁾ But, if one wishes to investigate the efficacy of biological response modifiers (BRMs) having an indirect antitumor effect mediated by host immune effector cells, the use of nude mice which are deficient in T-cell function is inappropriate. Therefore, we used the hepatic metastasis model produced by Tominaga *et al.*¹⁾ by the inoculation of mouse colon-26 tumor cells into the portal vein via the superior mesenteric vein.

Recently, *in vitro* and *in vivo* antitumor effects of TNF have been reported in many papers.^{7,14)} Most of these papers deal with antiproliferative effects on tumors transplanted into the subcutaneous tissues or other organs, as described by Nakata *et al.*, who reported the efficacy of TNF and anticancer drugs on colon-26 tumors.¹⁵⁾ The only report on the preventive effect on hepatic metastases is that of Brunda *et al.*, who reported that both rHuIFN- α A/D and rMuIFN- γ prevented liver metastases of M5076, a reticulum-cell sarcoma, transplanted into C57BL/6J mice.¹⁶⁾ Moreover, our present report is the only one to deal with the effects of pretreatment with TNF.

Prior to initiation of this experiment, we first studied the direct cytotoxicity of this nHuTNF- α against mouse colon-26 tumor cells by MTT assay *in vitro*, and found that this cell line is insensitive (data not shown). The results presented in Table I indicated a lack of effect of nHuTNF- α when the treatment with this preparation started immediately after tumor cell inoculation. In addition to the results of MTT assay, this suggested that nHuTNF- α was unable to act directly on circulating

tumor cells and subsequently to kill them. Gorelik *et al.* reported that circulating tumor cells were efficiently eliminated mainly by NK cells within 24 h after the tumor inoculation and that tumor metastasis was facilitated by the suppression of NK cellular activity.¹⁷⁾ We therefore supposed that the ineffectiveness of nHuTNF- α found in the present study might be due to the decrease of NK cellular activity following the surgical procedure on the mice. In addition, because TNF is well known to elevate procoagulant activity,¹⁸⁾ TNF might promote aggregation between tumor cells and platelets within the microvasculature of the liver and subsequently enhance the number of metastases.

As shown in Table III, pretreatment with nHuTNF- α caused a remarkable reduction in the number of metastatic tumors, and this effect was blocked by anti-asialo GM1 antibody. In recent studies, Talmage *et al.* showed that no augmentation of the NK activity of the splenic cells of C3H/HeN mice occurred 1 to 3 days after the single intravenous administration of rHuTNF- α .¹⁹⁾ According to our preliminary work, when administration of nHuTNF- α was started from 5 days before the inoculation and continued daily up to day 4 after the inoculation (total of 10 injections), a significant inhibition of hepatic metastases was not observed (data not shown). It can be speculated that comparatively long-term administration of TNF would induce the augmentation of asialo GM1-positive effector cells, mainly blood-borne NK cells, and these effector cells would act directly on tumor cells.

Concerning the NK cells lying in the liver, Wiltout *et al.* demonstrated that such BRMs as pyran copolymer maleic anhydride divinyl ether (MEV-2) augmented NK activity in blood, spleen, liver and lung, and that augmented organ-associated NK activity in the lung and liver was more resistant to depletion by the standard regimen of anti-asialo GM1 treatment than was NK activity in the blood and spleen.²⁰⁾ As shown in Fig. 1, histological examination of liver specimens in mice pretreated with nHuTNF- α revealed that there was a considerable lymphocyte infiltration around the tumor nodules, and most of them proved to be asialo GM1-positive. The number of these asialo-GM1-positive cells was significantly increased in comparison to that in the control group. This suggested the possibility that organ-associated NK cells might be augmented, and inhibit metastasis formation during the extravasation and/or early postextravasation phase of the metastatic process. Therefore, work is in progress to examine whether the levels of NK cell activity in the liver are enhanced.

Recently, some reports have appeared on the mechanism of the antiproliferative effects of TNF. Shimomura *et al.* reported that TNF impaired microcirculation by producing fibrin-like substances in newly formed micro-

capillaries within tumors, and that the antitumor activity of TNF depended upon a fully developed fine network of induced capillaries in the tumor.²¹⁾ Our results in Table II would be in agreement with their observation since nHuTNF- α would act at the stage when a tumor was still very small, though the time when the colon-26 tumor forms microcapillaries is unknown. On the other hand, local NK cells in the liver seemed not to be involved in the antiproliferative effects of TNF on the basis of our immunohistological studies. Because TNF is also reported to activate macrophages, other effector cells are probably involved in the antiproliferative effects of TNF. Therefore, we are now investigating NK cell activity, LAK cell activity and the functions of other effector cells.

Further investigation of other defence mechanisms against the metastatic process and combination therapy

with anticancer drugs, interleukin-2 and interferons in order to more effectively prevent hepatic metastases will be needed in the future.

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