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The Inhibition of Neoplastic Cell Proliferation with Human Natural Tumor Necrosis Factor

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Purified human natural tumor necrosis factor (n-TNF) was prepared by stimulating human leukemic B cell line (BALL-1) with Sendai virus. The colony formations of all of 18 human cancer-derived abnormal cell lines were suppressed by 10^3–10^6 U/ml of n-TNF, while n-TNF was nontoxic to all human normal fibroblast cells. This *in vitro* inhibition of cell growth was reversible. In breast adenocarcinoma MCF7 cells treated with n-TNF a specific decrease of DNA synthesis was observed, and DNA histograms showed a block at G_1 in the cell cycle. *In vivo* studies revealed that n-TNF suppressed the tumor growth of murine Meth A sarcoma, human renal adenocarcinoma (ACHN), malignant melanoma (SK-MEL-28) and glioblastoma (U-373 MG). Isobologram analysis showed that n-TNF synergistically inhibited cell growth in combination with human natural interferon (IFN)-α. *In vivo* synergism of n-TNF and IFN-α was also found in the U-373MG tumor model implanted into nude mice.

Key words: Tumor necrosis factor — Interferon — Synergism

Human cells produce many kinds of cytokines in response to many types of stimuli such as endotoxin, virus, bacteria and so on. Tumor necrosis factor (TNF) was found as a kind of cytokine which showed *in vitro* L cell killing activity and *in vivo* hemorrhagic necrotic activity on murine Meth A sarcoma implanted into mice.[1] It was originally thought that TNF was secreted from monocyte/macrophage cell population.[2,3] However, Rubin *et al.*[4] recently found that a hematopoietic-derived Luk II cell line secreted TNF on stimulation with a tumor promoter, mezerein. They purified two types of human natural TNFs with molecular weights of 70,000 daltons and 19,000–25,000 daltons by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The amino acid sequence and cDNA sequence of both rabbit TNF from macrophage-like cells[5] and human TNF from monocyte-like cells[6] have recently been determined.

We have found that a human leukemic B cell (BALL-1 cell) line[7] secreted human natural TNF (n-TNF) into the culture fluid together with human natural interferon (IFN)-α on stimulation with Sendai virus. The crude n-TNF in the culture fluid was purified to homogeneity on SDS-PAGE in order to determine the profiles of the *in vitro* antiproliferative and *in vivo* antitumor activities.

Materials and Methods

Cell Lines MCF7[8] was supplied by Dr. M. Namba (Kawasaki Med. Sch.), MKN-1[9] and MKN-28[9] were from Dr. T. Motoyama (Niigata Univ. Sch. Med.), HEC-1C[10] was from Dr. T. Kuwata (Chiba Univ.), KP1[11] was from Dr. K. Tanaka (Kyushu Univ.), SEKI-F[12] was from Dr. M. Sekiguchi (Inst. Med. Sci., Univ. Tokyo), Sk-MEL-28[13] was from Dr. F. Takaku (Tokyo Univ.), U-373MG[14] was from Otsuka Pharm. Co., Ltd., and HF8209[15] and HF8211 were from Dr. J. Imanishi (Kyoto Pref. Univ. Med.). M08 was established by the authors. A549,[16] A-498,[16] BT-20,[17] SK-LU-1,[18] HT-144,[18] SK-CO-1,[18] HeLa,[19] ACHN,[20] U-138 MG[14] and KB[21] were supplied by the American Type Culture Collection. BALL-1 cells, which was used for n-TNF production, were established from a patient with acute lymphoblastic leukemia by Miyoshi *et al.*[7] in 1976. BALL-1 cells were negative for EBNA, virtually 100% surface Ig-positive and human thymus-related antigen-negative.

Culture Media, Animals and Reagents MEM, methionine-free MEM, Ham's F-12 and RPMI

1640 were purchased from Nissui Seiyaku Co., Ltd., Japan. Dulbecco's modified MEM (D-MEM) was purchased from Life Technologies, Inc., USA. L-15 medium and non-essential amino acid mixture were purchased from Flow Laboratories, Inc., USA. All sera were obtained from Irvine Scientific, USA, through Nippon Bio-Supply Center, Japan. The 15.5 mmϕ well dishes, 35 mmϕ Petri dishes and 96-microwell plates were purchased from A/S Nunc, Denmark and 60 mmϕ Petri dishes from Corning Glass Works, USA. Mitomycin C (MMC), fluorouracil (5-FU) and doxorubicin hydrochloride (DXR) were purchased from Kyowa Hakko Kogyo Co., Ltd., Japan, and nimustine hydrochloride (ACNU) was purchased from Sankyo Co., Ltd., Japan. [^{3}H]Thymidine, [^{3}H]uridine, [^{35}S]methionine and Bolton-Hunter reagent for protein iodination (^{125}I) were supplied by Amersham International plc. UK. RNase and propidium iodide were purchased from Sigma Chemical Co., USA, and pronase P was obtained from Kaken Pharmaceutical Co., Japan. CBF$_1$ mice and BALB/c *nu/nu* mice were purchased from Shizuoka Laboratory Center, Japan and Nippon Clea, Japan, respectively.

The purified human natural IFN-α was prepared from the culture fluid of BALL-1 cells stimulated with Sendai virus by the authors as described by Imanishi *et al.*[22] and Tanimoto.[23] Sendai virus was also prepared by the authors.[24]

Sepharose gel coupled with murine anti-IFN-α monoclonal antibody (NK-2 Sepharose gel) was purchased from Celltech Ltd., UK. An ultrafiltration membrane (AIP-3013, MW 6000-cut) was purchased from Asahi Chemical Industry Co., Ltd., Japan. Phenyl-Sepharose gel and Sephadex G-200 gel were obtained from Pharmacia, Sweden. Murine anti-n-TNF monoclonal antibody (3D6 antibody) was prepared by the authors (unpublished).

n-TNF Preparations BALL-1 cells suspended in RPMI 1640 (5×10^6 cells/ml) were stimulated overnight with about 100 HAU/ml of Sendai virus at 35°. The cells were discarded by centrifugation and the supernatant was concentrated by using an ultrafiltration membrane (AIP-3013). The concentrated solution containing n-TNF was applied to a phenyl-Sepharose column. n-TNF was then eluted with 60% ethylene glycol in 0.01*M* sodium phosphate-buffered saline (PBS), followed by dialysis against PBS. The partially purified n-TNF solution was passed through an NK-2 Sepharose gel column to remove IFN-α. n-TNF was purified to homogeneity by affinity chromatography on a 3D6 antibody coupled-Sepharose gel column by eluting with 0.035*M* ethylamine, followed by gel filtration on a Sephadex G-200 column with ×2 PBS (maned fraction No. H-7-1). The purified n-TNF showed a single homogeneous band with a molecular weight of 17,000 to 18,000 daltons on SDS-PAGE by the method of Laemli.[25] Protein sequencing and cDNA sequencing of n-TNF[26] indicated it to be almost identical with human TNF secreted from a premyelocytic leukemic cell (HL-60) line.[6] All *in vitro* and *in vivo* experiments in this study were carried out by using purified n-TNF preparations with a specific activity of about 10^9 U/mg protein.

Titration of n-TNF was carried out by measuring the cytopathic effect (CPE) against murine L_{929} cells, which were subcultured in MEM supplemented with 10% bovine serum in a 96-microwell plate (1×10^4 cells/well). Actinomycin D (1.6 μg/ml final concentration) and n-TNF were simultaneously added to all microwells and the plate was incubated overnight at 37°. The final concentration of n-TNF giving half CPE was arbitrarily defined as 50 Laboratory units/ml. A Japan Standard Reference for TNF was recently supplied by Dr. S. Yamazaki, NIH, Japan, and 1 JRU was equivalent to 350 Laboratory units.

***In vitro* Cell Proliferation Inhibition** Table I shows the compositions of media employed for *in vitro* colony formation. One hundred to three thousand cells of each cell line were inoculated in 35 mmϕ or 60 mmϕ Petri dishes and cultured at 37° under 5% CO_2/95% air for 7–13 days either in the presence or absence of n-TNF, followed by Giemsa staining.[27] The number of colonies consisting of more than 20 cells was counted in order to calculate the n-TNF concentration which decreased colony formation by 50% (IC_{50}).

In order to clarify whether or not the antiproliferative activity of n-TNF was reversible, MCF7 cells were cultured by the mass culture method in the presence of 10^4–10^5 U/ml of n-TNF for 2 days, then culture was continued in fresh medium without n-TNF for another 7 days.

Isobologram analysis of the combination of n-TNF with human IFN-α or chemotherapeutics such as MMC, 5-FU, DXR and ACNU was performed as described by Steel and Peckham.[28] In the case of HEC-1C cells, which are totally resistant to human IFN-α, the combination effect of n-TNF (0 or 350 U/ml) and IFN-α (0, 10^3, or 10^4 IU/ml) was analyzed in triplicate at each concentration by the method of Dannecker *et al.*[29]

The effects of n-TNF on DNA, RNA and protein syntheses were also investigated by using MCF 7 cells cultured in the presence of 10^6 U/ml of n-TNF for 24 hr according to the method of Fuse and Kuwata.[30] The DNA histogram of MCF7 cells treated with 10^6 U/ml of n-TNF was analyzed for 36 hr in a fluorescence-activated cell sorter (FACS IV, Becton Dickinson Immunocytometry Systems, USA) by the method of Crissman *et al.*[31] n-TNF

protein was iodinated by the method of Littman *et al.*[32]

***In vivo* Antitumor Animal Experiments** The *in vivo* experiments were carried out with murine Meth A sarcoma intradermally (id) implanted into CBF_1 mice, and human glioblastoma (U-373MG), human renal adenocarcinoma (ACHN) and human malignant melanoma (SK-MEL-28) which were subcutaneously (sc) implanted into BALB/c *nu/nu* mice. The n-TNF was intravenously (iv) or intratumorally (itu) administered either alone or in combination with human natural IFN-α.

Results

***In vitro* Experiments** As shown in Table I, the colony formation of all of 18 human cancer-derived abnormal cell lines was inhibited by n-TNF but that of three normal fibroblasts was not suppressed. The results also show that the *in vitro* inhibition spectra against the 18 abnormal cell lines did not show any specificity regarding the origin of the tumors from which the cell lines had been established.

MCF7 cells, which were most sensitive to n-TNF *in vitro*, were employed in order to clarify how n-TNF would affect the intracellular macromolecule synthesis. Figure 1 indicates that over a 24 hr period, 10^6 U/ml of n-TNF specifically suppressed DNA synthesis and brought about very little decrease of RNA and protein syntheses. As shown in Fig. 2, the DNA histogram analysis revealed that control (untreated) MCF7 cells constantly divided during 36 hr under these experimental conditions. On the other hand, a major portion of S-, G_2- and M-phase cells had disappeared almost completely after a 24 hr

Table I. *In vitro* Antiproliferative Activity of n-TNF against Twenty-one Human Cell Lines

Cell line	Origin	IC_{50} (U/ml)	Culture media[a]
A. Extremely sensitive group			
MCF7	Breast adenocarcinoma	2.8×10^1	A
A-498	Renal carcinoma	3.0×10^1	D
A549	Lung carcinoma	4.3×10^1	A
B. Highly sensitive group			
U-373MG	Glioblastoma	1.8×10^2	G
SEKI-F	Malignant melanoma	2.5×10^2	F
BT-20	Breast carcinoma	2.7×10^2	D
HeLa	Uterine cervical carcinoma	3.0×10^2	A
U-138MG	Glioblastoma	4.5×10^2	E
HT-144	Malignant melanoma	4.7×10^2	E
SK-LU-1	Lung adenocarcinoma	5.4×10^2	B
C. Sensitive group			
SK-MEL-28	Malignant melanoma	1.0×10^3	D
MKN-1	Stomach adenocarcinoma	1.1×10^3	C
HEC-1C	Uterine adenocarcinoma	2.4×10^3	A
ACHN	Renal adenocarcinoma	4.0×10^3	A
KP1	Renal pelvic carcinoma	4.5×10^3	D
MKN-28	Stomach adenocarcinoma	8.3×10^3	A
KB	Oral epidermoid carcinoma	4.0×10^4	A
SK-CO-1	Colon adenocarcinoma	2.3×10^5	B
D. Resistant group			
HF8209	Normal diploid fibroblast	$1.0\times10^6<$	E
HF8211	Normal diploid fibroblast	$1.0\times10^6<$	E
M08	Normal diploid fibroblast	$1.0\times10^6<$	E

The colony count was carried out by using a colony counter (Colony Analyzer CA-7, Orient Instruments Ltd., Japan).

a) A, 10% fetal bovine serum/D-MEM; B, 10% fetal bovine serum/MEM; C, 10% fetal bovine serum/RPMI 1640; D, 10% fetal bovine serum/Ham's F12; E, 20% fetal bovine serum/L-15; F, 20% fetal bovine serum/MEM; G, 20% fetal bovine serum/MEM/non-essential amino acids/2m*M* sodium pyruvate. All media were supplemented with 10m*M* HEPES.

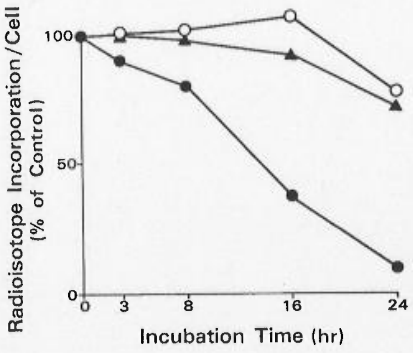

Fig. 1. Inhibition of DNA, RNA and protein syntheses of MCF7 cells. ●, DNA; ○, RNA; △, protein. Cells were inoculated in 15.5 mmϕ well dishes at a concentration of 4×10^4 cells/ml/dish in D-MEM supplemented with 5% bovine serum, and cultured for 3 days at 37°. The culture was continued for another 0, 3, 8, 16 and 24 hr in the presence of 10^6 U/ml of n-TNF. At the indicated time, the medium was changed to fresh methionine-free MEM containing 10^6 U/ml of n-TNF, and the cells were pulse-labeled for 1 hr with [^{3}H]thymidine (0.55 μCi/well), [^{3}H]uridine (5.5 μCi/well) or [^{35}S]methionine (5.5 μCi/well) as described in "Materials and Methods." The isotopic count in trichloroacetic acid-insoluble fractions was measured in a liquid scintillation counter (Liquid Scintillation System LSC-700, Aloka). The figures indicate the relative values as compared with control groups at each time.

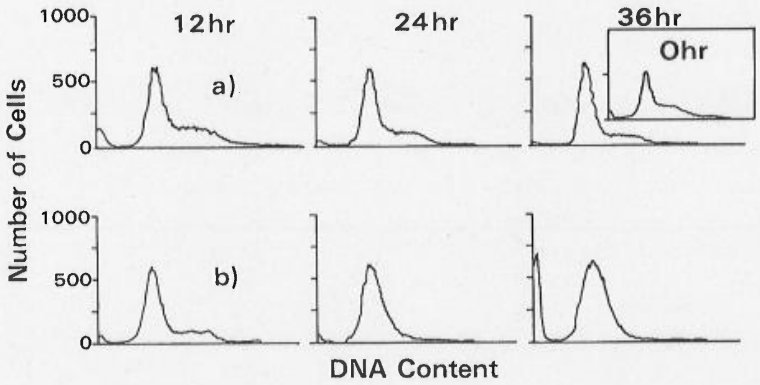

Fig. 2. DNA histogram of MCF7 cells treated with n-TNF. (a) Control (b) 10^6 U/ml. Cells were inoculated in 60 mmϕ Petri dishes at a concentration of 2.5×10^5 cells/10 ml/dish in D-MEM supplemented with 5% calf serum, and cultured for 2 days at 37°. The cells were treated with n-TNF for another 12, 24 or 36 hr. At the indicated time, cells were detached from the dishes with 0.25% pronase P, treated with 1% sodium azide and fixed with 70% ethanol. They were stocked at −20° until analysis. Flow cytometric analysis was conducted after treating the cells with 50 μg/ml of propidium iodide and 1 mg/ml of RNase as described in "Materials and Methods."

treatment with 10^6 U/ml of n-TNF. These results strongly suggest that n-TNF mainly blocked the G_1-phase of the cell cycle.

Figure 3 shows that n-TNF also inhibited the cell growth of MCF7 cells in the mass culture method, and immediate reproliferation of MCF7 cells was observed on changing to fresh culture medium without n-TNF at a growth rate similar to that of the control cells.

Isobologram analysis indicated clear synergistic effects of n-TNF and human IFN-α or ACNU in the inhibition of colony formation, as shown in Fig. 4. It was also observed that 5-FU and MMC inhibited synergistically, and DXR inhibited additively the cell proliferation of ACHN cells in the presence of n-TNF

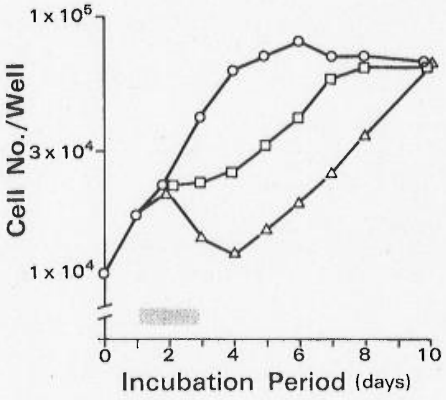

Fig. 3. Reversible inhibition by n-TNF of the cell proliferation of MCF7 cells. ○, Control; □, 10^4 U/ml; △, 10^5 U/ml. Cells were inoculated into a 96-microwell plate at a concentration of 0.2×10^4 cells/0.2 ml/well in D-MEM supplemented with 5% bovine serum. One day later, n-TNF was added to give a final concentration of 10^4 or 10^5 U/ml, and the culture was continued for a further 2 days (indicated by hatched bars). After the 2-day treatment with n-TNF, cells were cultured for another 7 days in fresh medium without n-TNF.

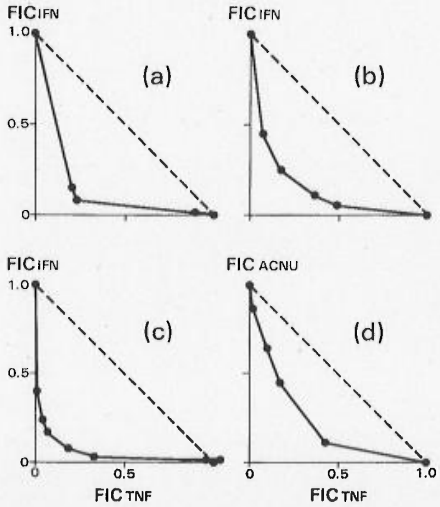

Fig. 4. Isobologram studies on the combination of n-TNF and human IFN-α (a, b, c) or ACNU (d). (a) Renal adenocarcinoma ACHN, (b) malignant melanoma SK-MEL-28, (c) glioblastoma U-373MG, (d) renal adenocarcinoma ACHN. Either 200 cells/2 ml for SK-MEL-28 cells or 500 cells/2 ml for ACHN and U-373MG cells was inoculated into 35 mmϕ dishes in the media listed in Table I and cultured for 7–9 days in the presence of n-TNF ($0–1 \times 10^4$ U/ml) and human IFN-α ($0–5 \times 10^2$ IU/ml) or ACNU (0–40 μg/ml). After Giemsa staining, the number of colonies was counted in order to obtain the fractional inhibitory concentration (FIC) for n-TNF (FIC_{TNF}) and human IFN-α (FIC_{IFN}) or ACNU (FIC_{ACNU}) by the method described in "Materials and Methods."

(data not shown). Interestingly, HEC-1C cells, which are totally resistant to IFN-α because they lack the IFN receptor,[10] also showed synergistic inhibition of colony formation by the combination of n-TNF and IFN-α.

Receptor analysis shows that all the cell lines including a normal fibroblast cell line expressed the n-TNF receptor on the cell surface (Table II). Even normal fibroblast cells, which are resistant to n-TNF, expressed the receptor on the cell surface with similar *Kd* value and receptor number to those of sensitive abnormal cell lines. We could not observe any correlation between n-TNF sensitivity and affinity/number of the receptor.

***In vivo* Experiments** The tumor mass increase of Meth A sarcoma was significantly inhibited by both iv and itu administration of n-TNF at doses of 2×10^5–2×10^6 U/mouse, as shown in Table III. In addition, a high frequency of complete tumor regression was observed in both itu and iv administration groups. The antitumor activity by the itu route, however, seemed to be 3 to 10 times stronger than that by the iv route.

Table IV shows that intratumoral administration of n-TNF also effectively suppressed the mass increase of human tumors implanted into BALB/c *nu/nu* mice. In particular, ACHN tumor was a very sensitive model; 5 out of 6 mice were completely cured by daily itu administration of 2×10^6 U/mouse. The combined effect of n-TNF and human IFN-α on U-373MG glioblastoma was also studied *in vivo* by daily itu administration (Table IV).

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