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Mechanism of the Combined Antitumor Effect of Natural Human Tumor Necrosis Factor-α and Natural Human Interferon-α on Cell Cycle Progression

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We have studied the mechanism of the synergistic effect of the combination of tumor necrosis factor-α (TNF-α) and interferon-α (IFN-α) on cell cycle progression using two-parameter flow cytometry *in vitro* and an immunohistochemical staining method *in vivo*. The cells used were human colon cancer cell line RPMI 4788 *in vitro* and *in vivo*, and human breast cancer cell line MX-1 and human renal cancer cell line NAMKO-1 *in vivo*. In the *in vitro* experiment, the cell cycle progressed normally as time elapsed in the control group. However, in the group treated with TNF-α and IFN-α in combination (combination group), it appeared that the transition from the S phase to the G_2/M phase was blocked, and the cells that accumulated in the S phase died. In the *in vivo* experiment with male nude mice of a CD-1 genetic background, the antitumor effect on all three kinds of cancer cells was significantly greater in the combination group than in the control group. The cell labeling index on staining with bromodeoxyuridine in the combination group became markedly larger and the mitotic index smaller than in the other groups. From these results, it was concluded that in the combination group, both *in vitro* and *in vivo*, tumor cells markedly accumulated in the S phase and their progression from the S phase to the G_2/M phase in the cell cycle was inhibited.

Key words: Tumor necrosis factor-α — Interferon-α — Cell cycle progression

Recent progress in the technology of genetic recombination has made highly purified cytokines available. Among such cytokines, tumor necrosis factor-α (TNF-α) has various physiological activities, such as endothelial cell procoagulant activity,[1] suppressing lipoprotein lipase activity,[2,3] stimulating collagenase and prostaglandine synthesis,[4,5] inducing fever,[6] osteoclast-mediated bone resorption,[7,8] activating neutrophils,[9] promoting growth of fibroblasts,[10,11] etc. It has been clarified *in vitro* and *in vivo* that the antitumor activity of TNF is synergistically enhanced in combination with interferons (IFNs)[12–17] and the combination has already been tried clinically.[18] The mechanism involved, however, has been only partially clarified.

In the present research, we studied the mechanism of the combination effect of natural human tumor necrosis factor-α (nHuTNF-α) and natural human interferon-α (nHuIFN-α) on the cell cycle progression of tumor cells. We analyzed the effects of these cytokines by two-parameter flow cytometry with propidium iodide (PI) and bromodeoxyuridine (BrdU) *in vitro*, and by immunohistochemical staining with BrdU *in vivo*.

The abbreviations used are: TNF, tumor necrosis factor; nHuTNF-α, natural human tumor necrosis factor-α; nHuIFN-α, natural human interferon-α; IFNs, interferons; PI, propidium iodide; BrdU, bromodeoxyuridine; HVJ, hemagglutinating virus of Japan; SDS, sodium dodecyl sulfate; i.v., intravenous or intravenously; LI, labeling index; MI, mitotic index.

MATERIALS AND METHODS

Cells RPMI 4788 cells derived from a human colon cancer were supplied by Roswell Park Memorial Institute, Buffalo, NY.[19] These tumor cells were maintained in RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (FBS, GIBCO) and used for *in vitro* and *in vivo* analyses. The RPMI 1640 medium supplemented with 10% FBS without drugs was used for control cultures.

MX-1 cells derived from a human breast cancer (infiltrating duct cell carcinoma, medullary tubular adenocarcinoma and estrogen receptor-negative) were supplied by Kyowa Hakko Co., Ltd. (Tokyo).[20]

NAMKO-1 cells derived from a human renal cell cancer (clear cell type) were established from lymphnode metastasis of renal cell cancer in our laboratory (Okayama University Medical School, Okayama).

Animals Male athymic specific-pathogen-free nude mice of a CD-1 genetic background obtained from Charles River Japan Inc. (Atsugi, Kanagawa) were used at 4 to 6 weeks of age. The mice were kept in sterilized cages equipped with an air filter and sterile bedding materials, and fed with sterilized water and food *ad libitum*.

Reagents nHuTNF-α and nHuIFN-α produced from HVJ (hemagglutinating virus of Japan)-stimulated BALL-1 cells (B-cell acute lymphatic leukemia line) and purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis were supplied by Hayashibara Biochemical Laboratories, Inc. (Okayama). The molecular weight of nHuTNF-α was 17,000.[21–23] The specific

activity was 3×10^6 JRU/mg protein. Our standard was titrated against a Japanese Reference (J-PS5KO 1, National Institute of Health, Tokyo). The titers of nHuIFN-α were determined by using a cytopathic effect inhibition assay using human FL cells challenged with sindbis virus, and standardized against the international reference preparation of human IFN-α (Ga 23-901-532). The reason why natural cytokines were used is because recombinant cytokines have different three-dimensional structures and less potent effects than natural cytokines.[24]

Microscopic observation *in vitro* RPMI 4788 cells were seeded in plastic plates (Falcon No. 3013, Falcon Labware, Oxnard, CA) at 5×10^3 cells/plate. Culture medium containing nHuTNF-α (1.4×10^{-5} mg/ml) and/or nHuIFN-α (1×10^4 IU/ml) was added after aspiration of the medium which was used for 12 h preculture at 37°C in 5% CO_2. Morphological changes were monitored by phase-contrast microscopy at 24, 48 and 72 h after the start of treatment. The bases of the concentration ratios of TNF-α and IFN-α were determined according to the previous studies.[14, 15]

Cell viability *in vitro* assay RPMI 4788 cells treated with 0.25% trypsin were dispersed on plastic plates (Falcon No. 3013, Falcon Labware) at 5×10^3/plate and cultured to standstill at 37°C in 5% CO_2. After 24 h of culture, the medium was removed by aspiration and culture medium containing nHuTNF-α (1.4×10^{-5} mg/ml) and nHuIFN-α (1×10^4 IU/ml) was added for 72 h of static culture at 37°C in 5% CO_2. The medium was replaced by fresh medium every 24 h. RPMI 1640 medium supplemented with 10% FBS without drugs was cultured as a control. Viable and total cell numbers were simultaneously counted at 24, 48 and 72 h after dyeing with 0.5% crystal violet by the dye uptake method.[25] Cell viability was expressed as percent according to the following equation:

$$\text{Cell viability } (\%)=\frac{\text{viable cells}}{\text{number of cells}}\times100.$$

Staining and measurement with PI *in vitro* RPMI 4788 cells were treated with nHuTNF-α (1.4×10^{-5} mg/ml) and/or nHuIFN-α (1×10^4 IU/ml) for 24, 48 and 72 h at 5×10^3/plate. After being suspended in EDTA and trypsin and fixed in 70% ethanol for 10 min, the cells were treated with RNase (Sigma Chemical Co., St. Louis, MO). They were washed twice, resuspended in 1 ml of PBS containing 5 μg/ml of PI and allowed to react for 30 min. Then the cells were measured by using a 580 nm long-pass filter, and the histogram of the results was analyzed with Dean's computer program.[26] The experiments were repeated at least three times.

Double staining with PI and BrdU RPMI 4788 cells were treated with control medium (plates 1 and 2) and with medium containing nHuTNF-α (1.4×10^{-5} mg/ml) and nHuIFN-α (1×10^4 IU/ml) (plates 3 and 4) for 60 h at 5×10^3/plate. Five μg/ml of BrdU (Sigma Chemical Co.) was added to the cells in each plate and incubation was carried out for 30 min in a CO_2 incubator at 37°C. The cells in plates 1 and 3 were washed twice in PBS, and resuspended in 100 μl of normal saline on ice. Two ml of cold 70% ethanol (−20°C) was added to the chilled suspension to fix the cells for 15 min. Two ml of 4 *N* HCl was then added to 2 ml of the fixed cell suspension and incubation was continued at room temperature for an additional 30 min. The cells were washed 3 times in PBS, and resuspended in 1 ml of 0.1 *M* $Na_2B_4O_7$, pH 8.5, to neutralize the acid. The cells were again washed twice, and resuspended in 0.5 ml of PBS. Twenty μl of Anti-BrdU FITC (Becton Dickinson) and 50 μl of 0.5% Tween 20/PBS were added and the cells were incubated for more than 1 h at 4°C. After being washed once, the cells were treated with RNase and washed again twice. The cells were resuspended in 1 ml of PBS containing 5 μg/ml PI and incubated for 15 min at 4°C. The single cells thus obtained were analyzed by two-parameter flow cytometry (Spectrum III, Ortho Diagnostics, Westwood).

The media of the remaining cells in plates 2 and 4 were removed by aspiration. Then the cells were washed twice in PBS, and treated again with control medium (plate 2) and with medium containing nHuTNF-α (1.4×10^{-5} mg/ml) and nHuIFN-α (1×10^4 IU/ml) (plate 4) for an additional 12 h. The cells of plates 2 and 4 were analyzed in the same ways, after the same procedures, as the cells in plates 1 and 3 described above.

Transplantation of tumor cells and evaluation of antitumor effects RPMI 4788 cells (4×10^6/0.2 ml cell suspension) were injected subcutaneously into the back of each mouse.[27] MX-1 and NAMKO-1 tumors were cut with scissors into blocks 2–3 mm^3. Each tumor line was inoculated subcutaneously into the backs of mice. The diameters of growing tumors were measured with calipers every 3 or 4 days. Drug efficacy against tumor cells was expressed as mean tumor weight. The tumor weight was calculated from the length (a) and width (b) of the tumors measured with calipers in millimeters according to the Battelle Columbus Laboratories Protocol. The calculation was done as follows:

$$\text{Tumor weight (mg)}=1/2\times a\times b^2.$$

The data were expressed as relative weight using the following formula.

$$\text{Relative tumor weight}=\frac{\text{mean tumor weight at a given time}}{\text{initial mean tumor weight}}$$

Drug treatment The tumors were allowed to grow for 1 to 2 weeks until the tumor size reached about 100 mm^3. Then drug injection into the tail vein of the nude mice was started. The mice were randomly allocated into 4

groups with at least 5 mice in each group. The drugs were injected into mice of all groups for 21 days as follows; a, saline (0.2 ml) intravenously (i.v.) (control group); b, nHuTNF-α (1.4×10^{-3} mg/mouse/day) i.v. (TNF-α group); c, nHuIFN-α (1×10^{6} IU/mouse/day) i.v. (IFN-α group); d, nHuTNF-α and nHuIFN-α (1.4×10^{-3} mg and 1×10^{6} IU/mouse/day, respectively) i.v. (combination group). Then the mice were killed for analysis of the tumor cells.

Analysis of cell cycle progression Mice were killed 1 h after the intravenous injection of 5 mg/kg of BrdU. The tumors were fixed in 70% ethanol, embedded in paraffin to be processed for histological examination, and stained by the avidin-biotin peroxidase complex (ABC) method using anti-BrdU antibody. The positive cells were counted with the aid of a dissecting microscope. Labeling index (LI) was defined as the ratio of the BrdU-positive cells to the total among 2,000 cells counted. Mitotic index (MI, the ratio of the mitotic cells) was calculated similarly.

Statistical analysis The statistical significance of differences was examined by using Student's t test.

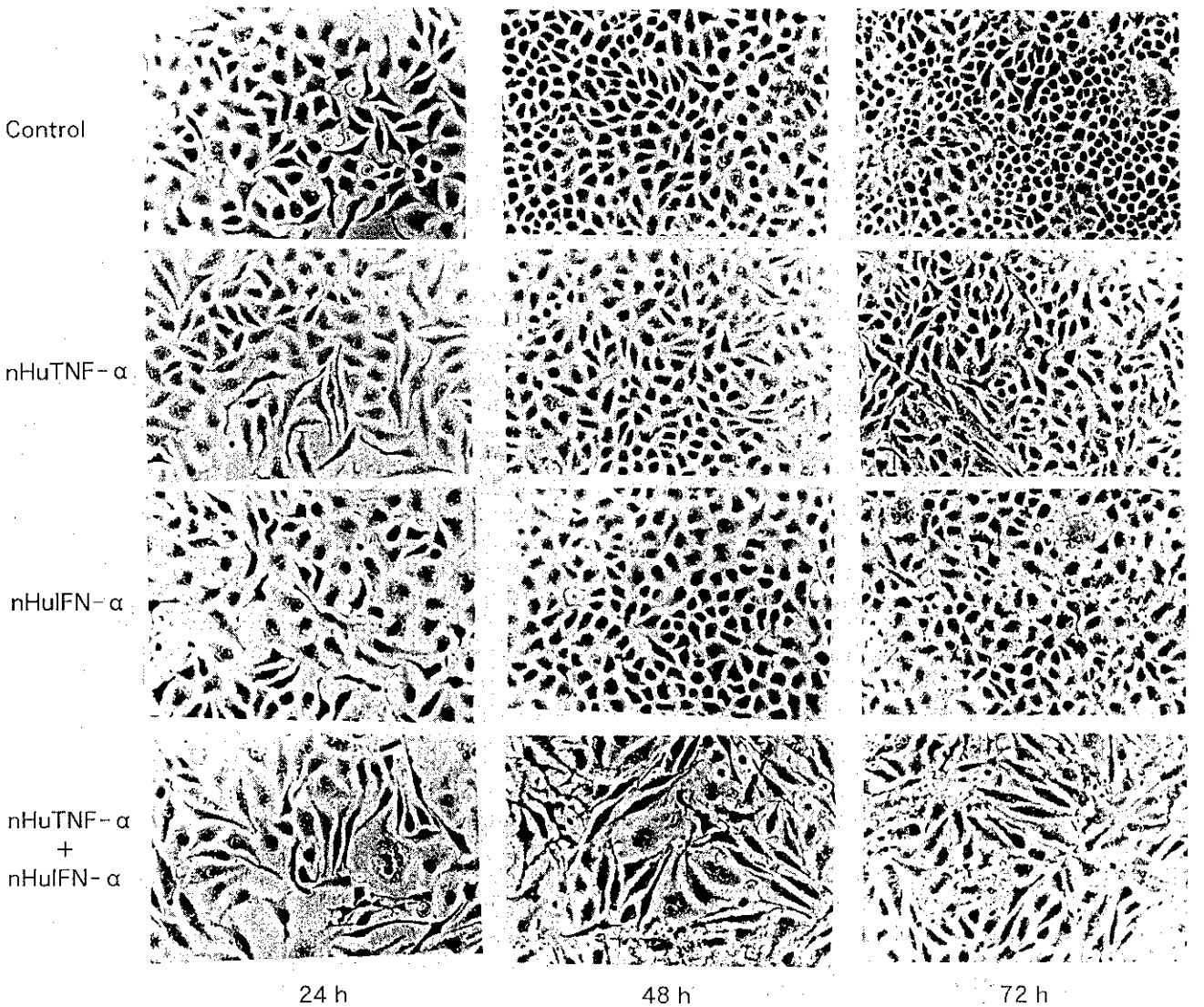


Fig. 1. Time course of the morphological change of RPMI 4788 cells with nHuTNF-α or/and nHuIFN-α. RPMI 4788 cells were seeded in plates at 5×10^{3} cells/plate and treated with control medium, nHuTNF-α (1.4×10^{-5} mg/ml) and/or nHuIFN-α (1×10^{4} IU/ml) for 72 h in static culture. The medium was exchanged at 24, 48 and 72 h. Morphological changes were monitored by phase-contrast microscopy after 24, 48 and 72 h of treatment. $\times200$.

RESULTS

Morphological change The results are shown in Fig. 1. As time passed, the cells grew, and binucleated and giant cells began to appear in the control group. In the TNF-α group, the tumor cells gradually changed to spindle-shaped during the 48 h treatment and this tendency was more marked during the 72 h treatment. In the IFN-α group, the cells changed little throughout the procedures. In the combination group, the tumor cells began to degenerate and enlarge during the 24 h treatment. In the 48 h treatment, the tumor cells became significantly degenerated and their appearance changed to spindle-shaped, vacuolated and ballooned. The increase in the cell numbers was markedly impaired. During the 72 h treatment, the tumor cells markedly changed and became more irregular in structure.

Tumor cell growth *in vitro* The results are shown in Fig. 2. While cell viability remained 100% during 72 h in the control group, it decreased as time passed in the combination group, with a marked decrease after 48 h. In addition, increase of the cell number was inhibited and a strong antitumor effect was noted *in vitro*.

Single staining with PI The ratio of the cells in the S phase to the whole cells in each group was as follows: for 24 h treatment, 36.2, 33.0, 40.1, 40.1%; for 48 h treatment, 31.3, 28.7, 43.4, and 45.4%; for 72 h treatment, 24.1, 34.0, 56.7 and 63.9%, in the control, TNF-α alone, IFN-α alone and combination groups, respectively (Table I). As time passed, the number of cells in the S phase increased, while the number of cells in the G_0/G_1 and G_2/M phase gradually decreased. The cell accumulation in the S phase began to be noted 48 h after the start of treatment in the TNF-α and IFN-α groups. As the cell cycle progressed, the cell accumulation in the S phase was markedly enhanced by the use of these cytokines in combination. This was consistent with the decrease of cell viability 48 h after the treatment, as shown in Fig. 2.

Double staining with BrdU and PI The course of cell accumulation in the S phase in the combination group

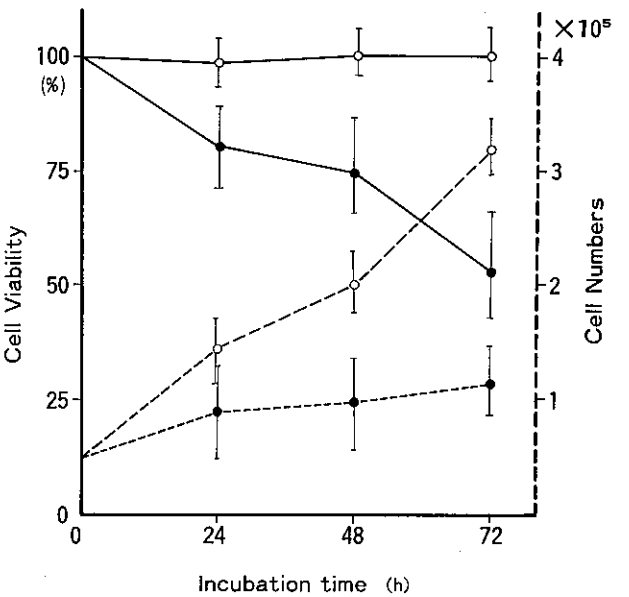


Fig. 2. Time course of the effect of nHuTNF-α and nHuIFN-α in combination. After 24 h culture of RPMI 4788 cells, the medium was removed by aspiration and culture medium containing nHuTNF-α (1.4×10^{-5} mg/ml) and nHuIFN-α (1×10^4 IU/ml) was added for 72 h in static culture. The medium was exchanged at 24, 48 and 72 h. Viable and total cell numbers were simultaneously counted at 24, 48 and 72 h after dyeing with 0.5% crystal violet by the dye uptake method. The experiments were repeated at least three times. (○) control; (●) nHuTNF-α and nHuIFN-α. Solid line, cell viability; dotted line, cell number; bars, SE

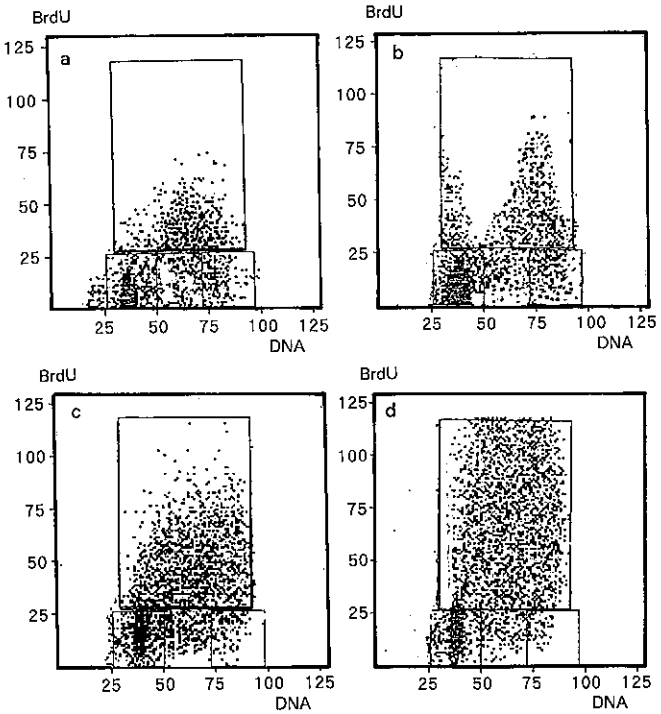
Table I. Cell Cycle Progression of RPMI 4788 Cells (Single Staining with PI)

Drug[a]	Cell cycle phase[b] (%)								
	24 h			48 h			72 h		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Control	54.1	36.2	9.7	60.8	31.3	7.9	66.2	24.1	9.7
nHuTNF-α	52.8	33.0	14.2	61.5	28.7	9.8	59.8	34.0	6.2
nHuIFN-α	54.6	40.1	5.3	52.6	43.4	4.0	40.0	56.7	3.3
nHuTNF-α+nHuIFN-α	56.8	40.1	3.1	51.0	45.4	3.6	34.2[c]	63.9[c]	1.9[c]

a) RPMI 4788 cells were treated with nHuTNF-α (1.4×10^{-5} mg/ml) and/or nHuIFN-α (1×10^4 IU/ml) for 24, 48 and 72 h.

b) RPMI 4788 cells were stained with propidium iodide (5 μg/ml) and measured by using a 580 nm long-pass filter. The histogram of the results was analyzed with Dean's computer program.

c) The differences between control and nHuTNF-α+nHuIFN-α, nHuTNF-α alone and nHuTNF-α+nHuIFN-α, nHuIFN-α alone and nHuTNF-α+nHuIFN-α were statistically significant by Student's t test ($P<0.05$).



was studied by double-staining with BrdU and PI (Fig. 3). In the control group, BrdU-positive cells in the G_0/G_1, G_2/M phase and BrdU-negative cells in the S phase increased 12 h after the start of culture in BrdU. This indicated that the cell cycle progressed from the S phase to the G_2/M, G_0/G_1 phase and from the G_2/M, G_0/G_1 phase to the S phase. In the RPMI 4788 cells in the combination group, BrdU-positive cells in the S phase

Fig. 3. Cell cycle analysis of RPMI 4788 cells by using BrdU and PI. RPMI 4788 cells were cultured with control medium, and were treated with nHuTNF-α (1.4×10^{-5} mg/ml) and nHuIFN-α (1×10^4 IU/ml) for 60 h. The medium was exchanged at the end of culture and stained with BrdU and PI, then the cells were treated again with nHuTNF-α and nHuIFN-α for an additional 12 h (total 72 h). The cells were analyzed by two-parameter flow cytometry at 60 and 72 h. a. Control culture for 60 h. b. Control culture for 72 h. c. Culture with nHuTNF-α and nHuIFN-α for 60 h. d. nHuTNF-α and nHuIFN-α for 72 h. The data are from a single experiment. The experiments were repeated at least three times. The upper box: the cells of BrdU positive cells and most of these cells are in the S phase. The left small box: most of the cells are in the G_0/G_1 phase. The right small box: most of the cells are in the G_2/M phase.

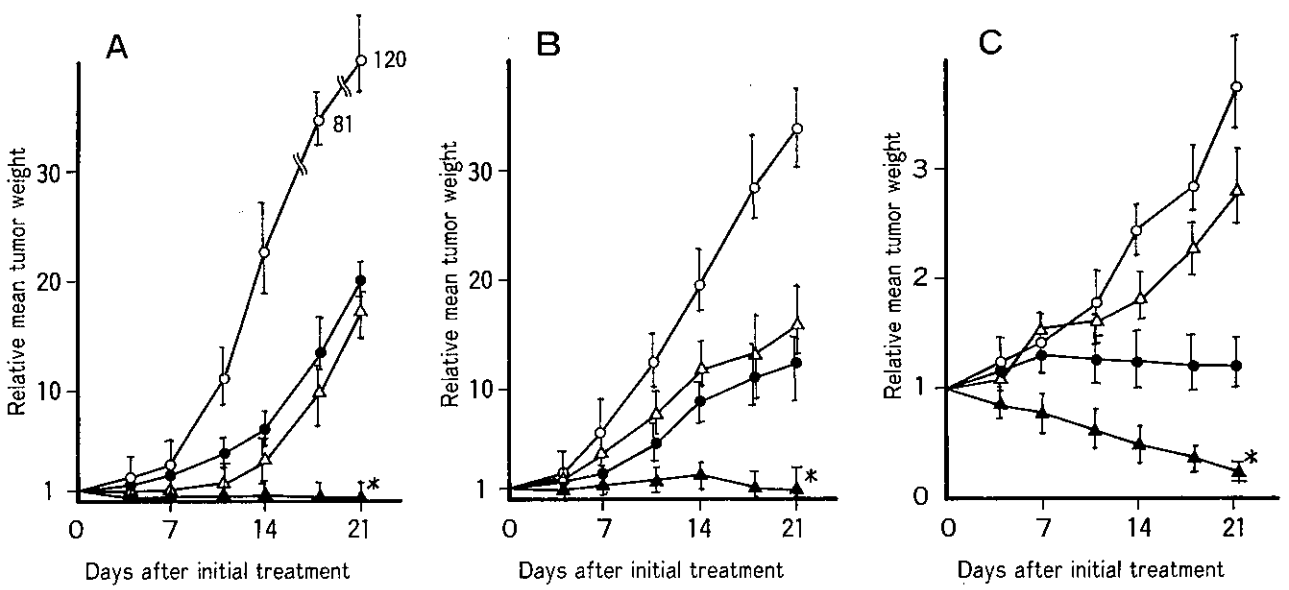


Fig. 4. Growth curves of human cancer cell line tumors subcutaneously inoculated in mice intravenously (i.v.) given nHuTNF-α or/and nHuIFN-α. a. Saline (control) i.v., 0.2 ml for 21 days (○). b. nHuTNF-α, 1.4×10^{-3} mg/mouse/day, i.v., for 21 days (△). c. nHuIFN-α, 1×10^6 IU/mouse/day, i.v., for 21 days (●). d. nHuTNF-α and nHuIFN-α, 1.4×10^{-3} mg and 1×10^6 mg/mouse/day, respectively, i.v., for 21 days (▲). * Differences between a and d, b and d, c and d at 21 days were statistically significant by Student's t test ($P<0.01$). Mice were used in groups of 6. A, RPMI 4788 (colon cancer); B, MX-1 (breast cancer); C, NAMKO-1 (renal cell cancer). Bars, SD.

markedly increased and neither BrdU-positive nor -negative cells were found in the G_0/G_1, G_2/M phase or in the G_2/M phase even 12 h after the start of culture in BrdU. This indicated that tumor cells were accumulated in the S phase, and cell cycle progression was arrested at this stage.

From this result, it is suggested that transition from the S phase to the G_2/M phase was blocked, and that the cells accumulated in the S phase died in the combination group. These results were consistent with the decrease of the cell viability at 48 h and afterward (Fig. 2).

Tumor growth *in vivo* No significant antitumor effect on RPMI 4788 or MX-1 cells was seen in the TNF-α or IFN-α groups compared with the control group (Fig. 4A, B). The antitumor effect on NAMKO-1 cells was not significant in the TNF-α group, but was significant in the IFN-α group compared with the control group. Tumor size or weight, however, did not change in these groups throughout the experiment (Fig. 4C).

On the other hand, the antitumor effects on all three kinds of tumor cells were significant in the combination group compared with the other three groups. Furthermore, the effects were different depending on the kind of tumor. The antitumor effect was greatest on the renal cell carcinoma (NAMKO-1) cell line among the three tumor cell lines examined.

Labeling index (LI) with BrdU and mitotic index (MI) The results are summarized in Table II. There were no significant differences of LI for any of the tumor cell lines among the control, TNF-α and IFN-α groups. However, the LI was significantly larger in the combination group than in the other three groups. As BrdU is an analogue of thymidine, it was taken into the newly synthesized DNA during the DNA-synthesizing period (S phase). Therefore, LI, the ratio of BrdU-positive cells to the total cells, indicates the proportion of cells in the S phase in the tissue. Thus, tumor cells were considered to have been accumulated in the S phase due to the treatment with nHuTNF-α and nHuIFN-α in combination.

There were no significant differences of MI in any of the three tumor cell lines among the control, TNF-α alone and IFN-α alone groups. On the other hand, MI was significantly smaller in the combination group than in the other three groups. This indicates that cell cycle progression from the S phase to the G_2/M phase was inhibited by treatment with nHuTNF-α and nHuIFN-α in combination.

Table II. Labeling Index with BrdU and Mitotic Index

Cell line	Drug[a]	Labeling index[b] (%)	Mitotic index[c] (%)
RPMI 4788	Control	31±3.8[d]	8.3±1.9
	nHuTNF-α	32±3.5	5.6±1.9
	nHuIFN-α	31±5.6	7.6±2.2
	nHuTNF-α+nHuIFN-α	47±3.1[e]	3.3±1.6[f]
MX-1	Control	43±4.3	5.4±1.9
	nHuTNF-α	40±2.1	3.6±0.7
	nHuIFN-α	36±5.0	5.2±1.9
	nHuTNF-α+nHuIFN-α	56±4.4[e]	2.3±0.7[e]
NAMKO-1	Control	26±4.5	9.7±1.7
	nHuTNF-α	32±3.3	7.0±0.4
	nHuIFN-α	34±2.6	7.7±1.7
	nHuTNF-α+nHuIFN-α	49±3.7[e]	2.4±0.4[e]

a) See the legend to Fig. 4.
b) Labeling index (LI) is the ratio of the BrdU-positive cells to the total for 2,000 cells counted.
c) Mitotic index (MI) is the ratio of the mitotic cells to the total for 2,000 cells counted.
d) Mean±SE.
e) The differences between control and nHuTNF-α+nHuIFN-α, nHuTNF-α alone and nHuTNF-α+nHuIFN-α, nHuIFN-α alone and nHuTNF-α+nHuIFN-α were statistically significant by Student's t test ($P<0.01$).
f) The differences between control and nHuTNF-α+nHuIFN-α, nHuTNF-α alone and nHuTNF-α+nHuIFN-α, nHuIFN-α alone and nHuTNF-α+nHuIFN-α were statistically significant by Student's t test ($P<0.05$).

DISCUSSION

TNF-α is known to show cell cytotoxic activity *in vitro*[28,29] and to cause hemorrhagic necrosis *in vivo*.[13,30-32] Fiers *et al.* have proposed that TNF binds with the receptor on the cell membrane, forms an endosome, and is taken into the cells. It stimulates the release of arachidonic acid, and then stimulates the release of a cell-fusing substance from the cells. The nature of this substance is unknown, but it may be superoxide or lipid peroxide.[33]

Many investigators have reported that IFN-α synergistically enhanced the antitumor activity of TNF-α.[12,13,16] We have reported synergistically enhanced antitumor effects on various cells *in vitro* and *in vivo* by combined treatment with nHuTNF-α and nHuIFN-α.[14,15,17] However, the mechanism of the synergistic antitumor effect is not fully understood. There have been reports claiming that IFNs increased the TNF-α receptors of the target cell, but this seems to have no relation to the synergistic effect.[34-36] There have not been many reports on the mechanism of TNF-α and IFN-α action from the viewpoint of cell cycle progression.

Ruff and Gifford have observed that TNF-α treatment increased RNA synthesis 6-fold, and cells appeared to be impaired after cell division.[37] Darzynkiewics *et al.* have reported that cell cycle progression was arrested at the G_2 phase when partially purified murine TNF-α was added

to L cells, as determined by flow cytometry. After marked G_2 phase accumulation for the first 4 h, tumor cells began to die 7 h after the addition and most of the cells had died by 24 h after the addition.[38] Creasey *et al.* have reported that IFNs delay cell transition from the G_0/G_1 to the S phase, and prolong the duration of the S phase.[39] Lee *et al.* have shown that neoplastic cells exposed to TNF-β (lymphotoxin) and human IFN-γ accumulated in the G_0/G_1 phase of the cell cycle.[25] These results suggested that cytokines affect nuclear division, not DNA synthesis.

We studied in detail the mechanism of the synergistic antitumor effects of nHuTNF-α and nHuIFN-α in combination using two-parameter flow cytometry with PI and BrdU *in vitro* and an immunohistochemical staining method with anti-BrdU antibody *in vivo*. In the combination group, RPMI 4788 cells were accumulated in the S phase and drastic changes of the cells were observed in the two-parameter analysis with PI and BrdU. The arrest of the cell cycle progression was also noted. The histogram pattern of the DNA at this time indicated the death of a cell group. This was consistent with the morphological observation by phase-contrast microscopy. As time elapsed, RPMI 4788 cells were accumulated in the S phase and the cell cycle progression from the S phase to the G_2/M phase was arrested in the combination group (Fig. 3). In the *in vivo* experiment, synergistic antitumor effects were seen on the RPMI 4788 cells (human colon cancer), MX-1 cells (human breast cancer) and NAMKO-1 cells (human renal cell cancer) in the combination group. Staining with BrdU revealed that the LI of the cells in the combination group tended to become markedly larger and MI smaller than in the other three groups (Table II). But the number of cells in the S phase after treatment with TNF-α and IFN-α *in vivo* was different from that *in vitro*. The difference between the *in vivo* and *in vitro* results may be attributable to the different experimental systems and different drug delivery systems. Accumulation of tumor cells in the S phase and the arrest of the cell cycle progression from the S phase to the G_2/M phase occurred in all three cancer cell lines that we tested *in vitro* and *in vivo* in the combination group. These results suggested that the synergistic antitumor effects of nHuTNF-α and nHuIFN-α in combination arise from an action on cell cycle progression.

Known antitumor cytokines include IFNs, TNF-α, TNF-β (lymphotoxin), interleukin-1 (IL-1), etc. TNF-α is identical with cachectin[40–42] and it has been used clinically for cancer treatment. However, high doses cause various side effects. If a synergistic antitumor effect can be expected with nHuTNF-α and nHuIFN-α in combination, it will improve the prospects for cancer therapy by allowing the use of lower doses of TNF-α, so reducing the side effects.

Cell accumulation in the S phase by using nHuTNF-α and nHuIFN-α in combination may also enhance the effectiveness of anticancer drugs which act at the S phase, such as 5-fluorouracil (5-FU).

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