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Hyperthermic Enhancement of the Antitumor Effect of Natural Human Tumor Necrosis Factor-α and -β: an *in vitro* and *in vivo* Study

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A synergistic antitumor effect of natural human tumor necrosis factor-β (TNF-β) in combination with hyperthermia was found, in comparison with that of TNF-α, using an *in vitro* antiproliferative assay on a human colon cancer cell line (RPMI4788) and an *in vivo* tumor growth inhibition assay on Meth A sarcoma cells. *In vitro* combined treatment with TNF-β (10,000 U/ml) and hyperthermia (at 43° for 60 min) synergistically inhibited the proliferation of the cells. Combined effects of TNF-α or natural human interferon-α or -γ (IFN-α, -γ) and hyperthermia were also examined, and furthermore, the combinations of TNFs and IFNs were examined in combination with hyperthermia at 42°; their antiproliferative effects were further augmented by hyperthermia. *In vivo* growth of Meth A sarcoma cells (5×10^5), transplanted subcutaneously into BALB/c mice, was inhibited significantly ($P<0.05$) with the combination of TNF-α or -β (2×10^5 U/mouse) and hyperthermia (at 43° for 60 min) as compared to either a single intravenous injection of TNF-α or -β alone or the hyperthermia alone. The influence of TNF-β and hyperthermia on the cell cycle was examined. Flow cytometric analysis showed that RPMI4788 cells treated with TNF-α or -β accumulated in the S phase of the cell cycle, and that hyperthermia (at 42° for 60 min) alone had no influence on the cell cycle and did not augment the S phase accumulation of the cells treated with TNF-α or -β.

Key words: Tumor necrosis factor-α — Tumor necrosis factor-β — Hyperthermia — Synergistic effect — Flow cytometry

Hyperthermia has attracted attention as a form of combined cancer therapy following the development of new heating systems. Nevertheless hyperthermia alone is not very effective against cancer cells Recently, not only recombinant type but also natural type TNF-α and TNF-β (human lymphotoxin) have been purified and produced in large quantities by the application of biotechnology. Recently it has been reported that the antitumor effect of TNF-α was augmented by incubation at increased temperatures.[1-4] But there has been no reports about the combined effect of TNF-β and hyperthermia.

In this report, in order to make a comparison between the augmented antitumor effect of TNF-β and that of TNF-α in combination with hyperthermia, we used a human colon cancer cell line (RPMI4788) and Meth A sarcoma, which were previously used in studies concerning the cell cycle analysis of TNF-α or the synergism of hyperthermia and TNF-α.[3,5] We found a synergistic antitumor effect of TNF-β and hyperthermia both *in vitro* and *in vivo*. The *in vitro* combined effect of TNF-α or -β and hyperthermia was examined by means of an antiproliferative assay using RPMI4788 cells. The synergistic effects of TNFs and IFNs are well known, and we also used IFN-α/-γ to examine the combined effects of hyperthermia and each combination of TNFs and IFNs in the hope of obtaining a stronger antitumor effect. *In vivo*, the combined effect of TNF-α or -β and hyperthermia was examined by means of a tumor growth inhibition assay using Meth A sarcoma in mice. On the other hand, in an effort to understand the mechanism of the increased combined effect of TNF-α or -β and hyperthermia, actively dividing asynchronous RPMI4788 cells were treated with TNF-α or -β and hyperthermia, alone or in combination, and then were subjected to analysis of cell cycle distribution by flow cytometry.

Abbreviations: TNF-α, -β, tumor necrosis factor-α, -β; IFN-α, -γ, interferon-α, -γ: HVJ, hemagglutinating virus of Japan; HT, hyperthermia.

Materials and Methods

Tumor Cells RPMI4788 cells derived from a human colon cancer were supplied by Rosewell Park Memorial Institute (Buffalo, N.Y.).[6] This cell line was maintained in RPMI1640 (Nissui Pharmacological Co., Tokyo) supplemented with 10% fetal calf serum (Gibco, N.Y.) at 37° in a humidified 5% CO_2 atmosphere. Cells in the exponential growth phase were harvested for the experiments. Meth A sarcoma cells were supplied by Chugai Pharmaceutical Co., Ltd., Tokyo.

Reagents TNF-α, TNF-β and IFN-α were produced from a B cell acute lymphatic leukemia line (BALL-1 cells) sensitized with hemagglutinating virus of Japan (HVJ), purified to over 99% homogeneity.[7-9] IFN-γ was produced from lipopolysaccharide-stimulated human myelomonocytic HBL-38 cells, purified to over 99% homogeneity.[10] These cytokines were kindly supplied by Hayashibara Biochemical Laboratories, Inc., Okayama. Titers of TNF-α and TNF-β were determined on the basis of cytolytic activity against actinomycin D-treated L929 mouse fibroblasts using a method developed for TNF or lymphotoxin by Eifel *et al.*[11] The amount giving a 50% reduction in cell viability compared to control values was used as the standard unit. Based on the assay results, the specific activity of TNF-α was 1×10^9 U/mg protein and that of TNF-β was 5×10^8 U/mg protein. Titers of IFN-α and -γ were determined in a cytopathic effect inhibition assay using human FL cells challenged with sindbis virus, and standardized against an international reference preparation of human IFN-α or -γ (Ga23-901-532, Ga23-901-530). The specific activity of IFN-α was 2×10^8 IU/mg protein and that of IFN-γ was 2.1×10^7 IU/mg protein.

Mice Six-week-old female BALB/c mice were obtained from CLEA Japan, Inc., Tokyo. Throughout the study, the mice were given food and water freely in our experimental animal center.

***In vitro* Assay** RPMI4788 cell suspensions were poured into glass test tubes in the presence of TNF-α, TNF-β, IFN-α (10, 100, 1000, 10,000 U·IU/ml), IFN-γ (0.01, 0.1, 1, 10 IU/ml) or medium only as a control. The tubes were warmed at 37°, 41°, 42° or 43° for 60 min in a water bath (Thermobox model A1 M100, Thermonics Co., Ltd., Tokyo). The tubes were held in a light wire frame to allow the water to circulate freely around them. The temperature inside the tube and outside in the bath water did not differ by more than 0.2°. Temperature was measured with a Termo Finer-N thermometer (Termo Co., Ltd., Tokyo). When the heating was finished, cells were seeded in triplicate into 96-well microtiter plates (Falcon Labware, Oxnard, Calif.) at a concentration of 5,000 cells/0.2 ml/well in the presence of each cytokine. After incubation at 37° in a humidified 5% CO_2 atmosphere for 4 days, the cells were stained with 0.5% crystal violet.[12] The plates were rinsed twice with PBS and air-dried. The dye was eluted with Sorenson's buffer (0.1*M* glycine and HCl, pH 3.0) in 30% ethanol, and the absorbance at 590 nm was determined with a plate analyzer, model FT-II (Toyo Sokki Co., Ltd., Tokyo). Viable cell numbers were estimated from the absorbance based on the correlation between this eluted dye and cell numbers.[13] These experiments were repeated at least three times. Cell growth was calculated according to the following formula:

$$\text{percent growth (\% growth)} = \frac{\text{mean absorbance of the treated group}}{\text{mean absorbance of the control group at 37°}} \times 100.$$

The mean and the standard deviation (SD) of the % growth were calculated and the mean % growth value of the wells treated with each combination of cytokine and hyperthermia were compared to the expected value calculated by multiplication of the mean % growth value of the wells treated with each cytokine alone by the mean % growth value of the wells treated with hyperthermia alone. If the difference between the experimental value and the expected value was more than 2SD, we judged the combined effect to be synergistic.[14] The combinations of TNF-α, -β and IFN-α, -γ with hyperthermia were also examined in the same way. The combination ratio per unit was as follows;

nHuTNF-α(-β) : nHuIFN-α = 1(1) : 1

nHuTNF-α(-β) : nHuIFN-γ = 1(1) : 0.01

***In vivo* Antitumor Effect** Viable murine Meth A sarcoma cells (5×10^5 suspended in 0.1 ml of PBS) were inoculated subcutaneously into the left thigh of BALB/c mice. Tumors were measured (length, width and height) with sliding calipers by the same person. After measuring the tumor, the tumor volume (V) was calculated using the following formula[15]:

$$V=\pi(a\times b\times c)\div 6$$

(a, length; b, width; c, height).

Treatment was administered on the 7th day after the tumor cell inoculation (day 0). Tumor-bearing mice were assigned randomly to test groups consisting of five mice each. The experimental groups were (a) control, (b) treated with hyperthermia, (c) treated with TNF-α, -β, (d) treated with hyperthermia and TNF-α, -β. TNF-α (2×10^4, 2×10^5 U/0.1 ml/mouse) or TNF-β (2×10^5 U/0.1 ml/mouse) was administered intravenously on day 0 (single injection). Hyperthermia was achieved by immersing the thighs of the mice in a water bath at 42° or 43° for 60 min after the administration of TNF-α, -β. The relative mean tumor volume (RV)

was calculated as Vi/Vo, where Vi is the mean tumor volume of a group at any given day and Vo is the mean tumor volume just before the treatment. The antitumor effects were evaluated in terms of Trv/Crv during the experiment, where Trv is the relative mean tumor volume of the treated group and Crv is the relative tumor volume of the control group at the same time. Comparisons of the RV of the combined treatment group with either the RV of the TNF-α, -β, group or that of the hyperthermia group were analyzed by Student's t-test, and if there was a statistically significant difference, we judged the combined effect to be synergistic.

Flow Cytometric Analysis Cellular DNA content of RPMI4788 cells treated with TNF-α, -β (10,000 U/ml) and/or hyperthermia (at 42° for 60 min) was measured by flow cytometry. Treated cells

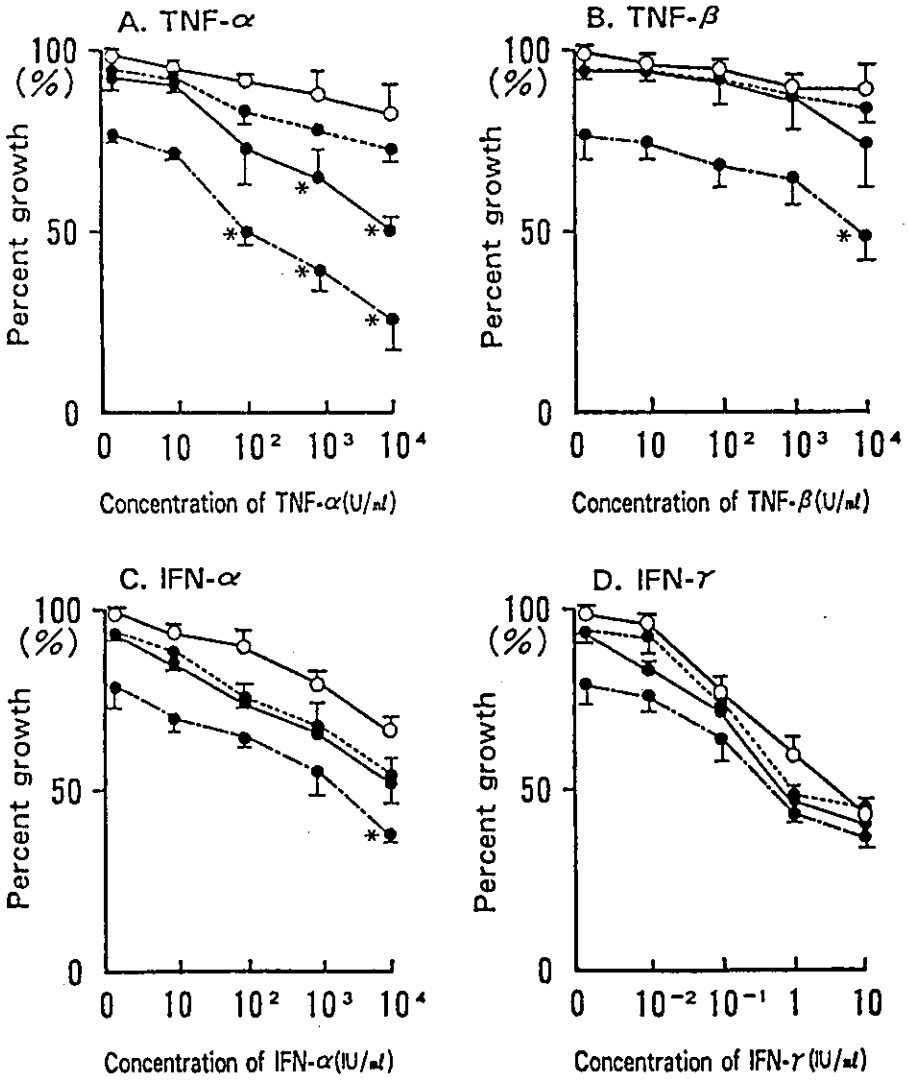

Fig. 1. *In vitro* antiproliferative activity of TNF-α, -β, IFN-α, -γ and hyperthermia in combination against RPMI4788 cells. Cells were warmed at 37°, 41°, 42° or 43° for 60 min in the presence of each cytokine and were seeded into microtiter plates at a concentration of 5,000 cells/well. Antiproliferative activity of each cytokine was determined by a dye uptake method after 4 days of incubation: ○ 37°; ●···●, 41°; ●—●, 42°; ●-·-●, 43°. Plots, mean±SD (bars); *, synergistic effect.

were seeded in a tissue culture plate (Falcon 3013) at 3×10^5 cells/plate and were fixed with 40% ethanol after incubation for 72 hr.[5] The cells were stained with propidium iodide by a rapid staining technique.[16] Fluorescence of individual cells stained with propidium iodide was measured with a Spectrum III flow cytometer (Ortho Diagnostics, Westwood, Calif.). Cell cycle phase distribution was analyzed by applying a computer model.[16] All of these experiments were performed at least three times.

Results

***In vitro* Assay** Antiproliferative activity of cytokines and hyperthermia (HT), alone or in combination, against RPMI4788 cells is shown in Fig. 1. The effect of each cytokine was augmented by combination with HT, and this effect was increased by further temperature elevation. The greatest enhancement by HT was observed with TNF-α, and the least

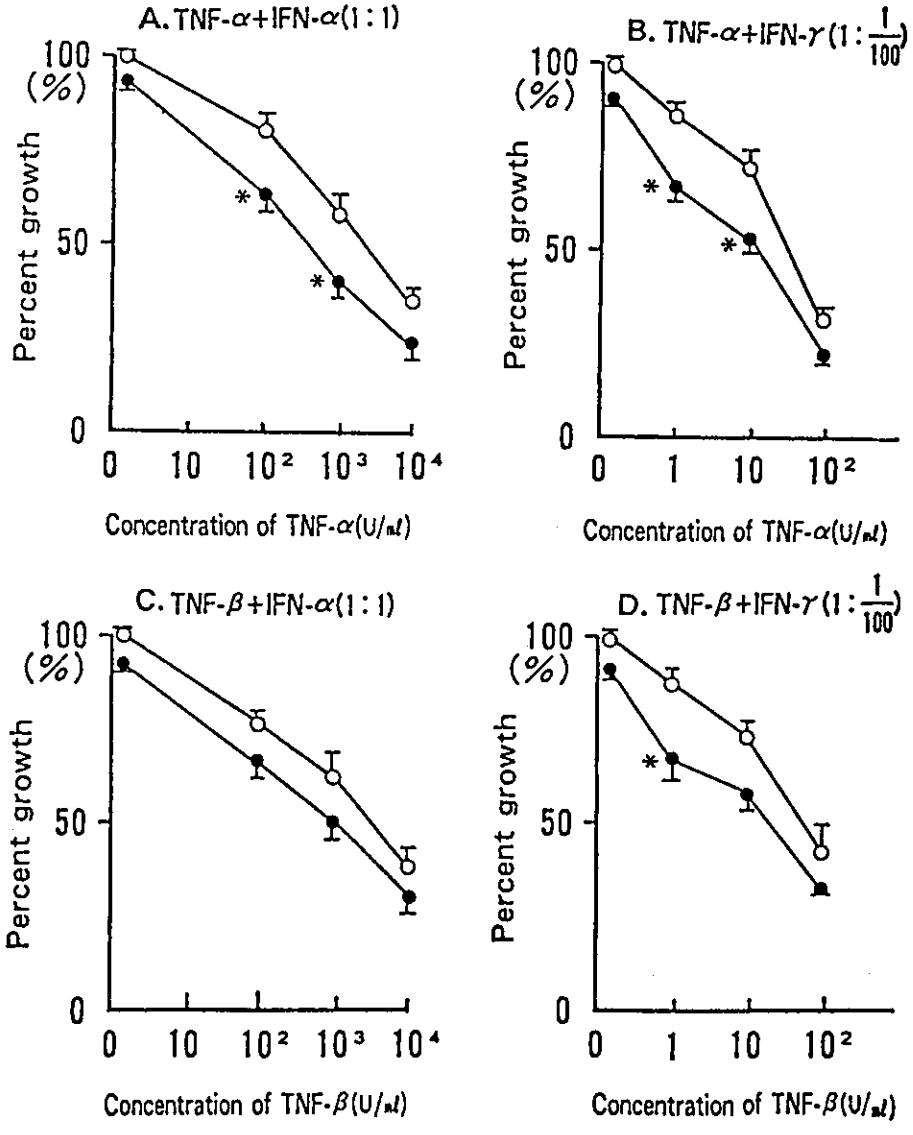

Fig. 2. *In vitro* antiproliferative activity of the combinations of TNF-α, -β and IFN-α, -γ and hyperthermia in combined therapy against RPMI4788 cells. All other conditions were as described in the legend to Fig. 1: ○, 37°; ●, 42°. Plots, mean±SD (bars); *, synergistic effect.

Table I. Antitumor Effects of TNF-α, -β and Hyperthermia in Combination on Meth A Cells Transplanted Subcutaneously in BALB/c Mice

Reagent	RV[a]	Trv/Crv[b] (%)
Control (saline)	4.91±0.94[c]	100.0
HT[d] (42°, 60 min)	3.70±1.00	75.3
HT (43°, 60 min)	3.79±0.94	77.2
TNF-α (2×10^4 U/mouse)	3.12±1.55	63.5
TNF-α (2×10^5 U/mouse)	2.08±1.19	42.4
TNF-α (2×10^4 U)+HT (42°)	2.55±1.10	51.9
TNF-α (2×10^4 U)+HT (43°)	1.75±1.43	35.6
TNF-α (2×10^5 U)+HT (42°)	1.83±0.34	37.3
TNF-α (2×10^5 U)+HT (43°)	0.10±0.15[e]	2.0
TNF-β (2×10^5 U/mouse)	3.40±1.38	69.2
TNF-β (2×10^5 U)+HT (42°)	2.09±1.11	42.6
TNF-β (2×10^5 U)+HT (43°)	0.17±0.33[e]	3.5

a) Relative mean tumor volume on day 8.
b) Comparison of the relative tumor volume between treated and control groups.
c) Mean±SD (n=5).
d) Hyperthermia.
e) Statistically significant ($P<0.05$) by Student's *t*-test as compared to either the hyperthermia group or the TNF-α, -β group.

with IFN-γ. Synergistic effects were shown by the combinations of TNF-α (1000 and 10,000 U/ml) and HT (42°), TNF-α (100, 1000 and 10,000 U/ml) and HT (43°), TNF-β (10,000 U/ml) and HT (43°) and IFN-α (10,000 IU/ml) and HT (43°), and increased with increasing concentrations of TNF-α. In a comparison of TNF-α and TNF-β, the antitumor activity of the combination of TNF-α (100 U/ml) with HT (43°) was almost equivalent to the combined effect of TNF-β (10,000 U/ml) and HT (43°). The effects of two cytokines, combinations of TNF-α, -β and IFN-α, -γ, with or without HT (42°) are shown in Fig. 2. The effects of the combinations of TNFs and IFNs were augmented by HT (42°). Synergistic effects were shown by the combination of HT with TNF-α and IFN-α, or with TNF-α, -β, and IFN-γ.

***In vivo* Antitumor Effect** Hyperthermia (42° and 43°) alone did not show a significant antitumor effect against Meth A sarcoma cells transplanted subcutaneously into BALB/c mice. The antitumor effect of TNF-α (2×10^4 and 2×10^5 U/mouse) alone was not significant but was augmented by HT (42° and 43°), and the combined antitumor effect of TNF-α and HT was significant at 43°. TNF-β (2×10^5 U/mouse) alone also did not show a significant antitumor effect, but the effect of TNF-β was augmented by HT (42° and 43°). A synergistic effect was shown in the combinations of TNF-α (2×10^5 U/mouse) and HT (43°) (Trv/Crv: 2.0%, $P<0.05$) and TNF-β (2×10^5 U/mouse) and HT (43°) (Trv/Crv: 3.5%, $P<0.05$) (Table I). During the experimental period, no significant body weight loss was observed in any treatment group (data not shown).

Flow Cytometric Analysis As shown in Fig. 3, no significant difference in the cell cycle distribution was observed between untreated cells and the cells treated with HT (42°) alone, but an increase in the percentage of cells accumulating in the S phase was observed in cells treated with TNF-α or -β (10,000 U/ml). When the cells were treated with the combinations of TNF-α or -β and HT, the accumulation in the S phase of the cell cycle was almost the same as in cells treated with TNF-α or -β.

Discussion

Hyperthermia has been applied to cancer therapy on the basis of the fact that the lethal thermal damage to cancer cells was found to increase significantly at temperatures over 42.5°.[17] It has already been reported, as the

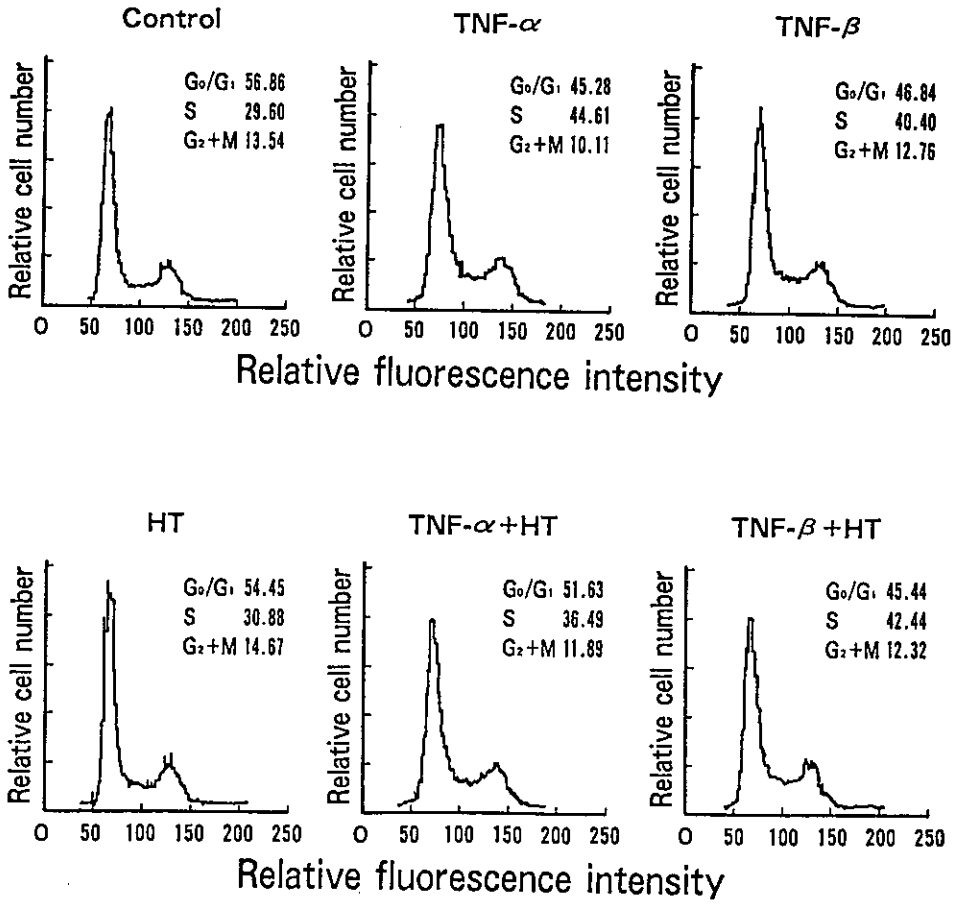

Fig. 3. DNA histogram and kinetic cell cycle analysis of RPMI4788 cells treated with TNF-α, -β (10,000 U/ml) and/or hyperthermia (42° for 60 min) at 72 hr after the start of incubation. The data are from a single experiment. The experiments were repeated at least three times. HT, hyperthermia.

mechanism of the antitumor effect of hyperthermia, that the thermosensitivity of cancer cells is augmented in the S phase of the cell cycle.[18] On the other hand, TNF-α and -β have antitumor activity against a variety of cancer cells. Technical developments have allowed us to produce highly purified cytokines in large quantities.

Recently, a few studies have appeared on the combination of cytokines and hyperthermia. Delbrück *et al.* first reported a pronounced increase in tumor inhibition by rat IFN in a rat osteosarcoma cell line with increased temperature.[19] Ruff and Gifford, and Watanabe *et al.* reported that an increase in TNF-α cytotoxicity was seen in L929 and L-M cells incubated at 39° to 40° for 6 hr, instead of 37°.[1,3] These mouse fibroblast cells were very sensitive to TNF-α, and the long incubation time was not suitable for hyperthermic treatment and could not be applied in practice. Moreover, there has been no study about the enhanced antitumor activity of TNF-β in combination with hyperthermia. On the other hand, the mechanism responsible for an increase of effects of cytokines and hyperthermia is not well understood. Groveman *et al.* reported that in a human bladder cancer cell line, an increase in temperature during IFN-α, -β treatment enhanced the levels of 2′-

5′A synthetase activity and suggested that an elevation in temperature may have a direct effect on the cell membrane, influencing receptor expression or affinity for cytokines.[20] Niitsu *et al.* reported that the synergistic cytotoxic effect between recombinant TNF-α and hyperthermia (incubation at 38.5° or 40°) was related to an accelerated turnover rate of TNF-α-receptor complex at elevated temperature rather than to changes in the number of cell receptors or binding strength.[4]

In this study, we showed that the *in vitro* and *in vivo* antitumor effects of TNF-β against RPMI4788 cells and Meth A sarcoma were augmented synergistically, like those of TNF-α, in combination with hyperthermia. However, *in vitro*, a difference in thermosensitivity between TNF-α and TNF-β was observed. In combination with hyperthermia at 43°, the antiproliferative effect of TNF-α was almost 100 times greater than that of TNF-β. On the other hand, the combination of IFNs and TNFs has recently been shown to achieve synergistic antitumor activity.[5, 13] It was shown that the activities of the combinations of IFNs and TNFs were augmented in combination with hyperthermia, and that the concentrations of IFNs and TNFs can be reduced to about one-tenth by the combination of hyperthermia (at 42° for 60 min) as compared with IFNs and TNFs alone for an equivalent effect. *In vivo*, a statistically significant ($P<0.05$) improvement was observed in the combination of TNF-β (2×10^5 U/mouse) and hyperthermia (at 43° for 60 min) as compared to either TNF-β alone or hyperthermia alone. We thus showed that TNF-β was very effective in combination with hyperthermia *in vivo*, as well as TNF-α.

Changes in the cell cycle distribution of cells have been examined by flow cytometry, and there have been several reports on the cell cycle of cells treated with TNF-α, but only one report concerning TNF-β. Darzynkiewics *et al.* reported that L cells treated with TNF-α showed cumulative arrest in $G_2 + M$.[21] Naomoto *et al.* reported that RPMI4788 cells, L929 cells and KB cells treated with TNF-α or IFN-α accumulated in the S phase, and that the accumulation in the S phase was augmented by the combination of TNF-α and IFN-α.[5] Lee *et al.* reported that B16 melanoma cells treated with TNF-β accumulate in the G_0-G_1 phase.[12] We thought that hyperthermia might enhance the effect of TNF-α or -β on the cell cycle, and that caused the synergism. It was shown that TNF-β accumulated RPMI4788 cells in the S phase. However, hyperthermia (at 42° for 60 min) did not augment the S phase accumulation of the cells treated with TNF-α or -β.

In conclusion, we obtained two new findings, i.e., the synergism of TNF-β and hyperthermia, and the accumulation in the S phase of the cell cycle of the cells treated with TNF-β. In addition, we propose that one of the mechanisms of the augmented antitumor effect of TNF-β or -α in combination with hyperthermia is that the S phase accumulation of the cells treated with TNF-β or -α enhances the sensitivity of the treated cells to hyperthermia, thus causing the augmented antitumor effect.

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