Jpn. J. Cancer Res. 83, 540–545, May 1992

Mechanism of Synergistic Cytotoxic Effect between Tumor Necrosis Factor and Hyperthermia

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We previously reported that recombinant human tumor necrosis factor (rhTNF) and hyperthermia had a synergistic effect against tumors, *in vitro* and *in vivo*. We have now investigated the mechanism of this synergy by measuring the lysosomal enzyme activity and hydroxyl radical production of L-M cells treated with rhTNF and/or hyperthermia. A synergistic activation of lysosomal enzyme and the induction of hydroxyl radical production in L-M cells treated with both rhTNF and hyperthermia was observed. A synergistic cytotoxic effect was observed when rhTNF and hyperthermia were combined, and was inhibited by the addition of a reactive oxygen scavenger, dimethyl sulfoxide or bipyridine. The results show that the augmenting effect of hyperthermia on lysosomal enzyme activation and induction of hydroxyl radical production by rhTNF plays an important role in the synergistic cytotoxic effect.

Key words: Tumor necrosis factor — Hyperthermia — Synergistic cytotoxic effect — Mechanism

Tumor necrosis factor (TNF) is a macrophage/monocyte-derived cytokine[1-3] that exhibits a potent antitumor activity against some types of tumor cells.[4-10]

We previously reported a synergistic increase in the antitumor effects of recombinant human TNF (rhTNF) and hyperthermia.[11,12] The mechanism of the synergistic antitumor effects on tumor cells, however, remains largely unexplained.

To increase the efficacy of this combination therapy in clinical practice, it would be important to determine the mechanism of the synergistic effects in order to establish the optimal timing of their combined use. In this study, assuming that the cytotoxic action of TNF is based on the direct lethal effect of lysosomal enzymes[13-15] and hydroxyl radicals (·OH),[16,17] we attempted to determine the mechanism of the synergistic effect of TNF and hyperthermia in combination by determining whether hyperthermia activates lysosomal enzymes and stimulates hydroxyl radical production. In addition, the optimal timing of TNF and hyperthermia in combination required for a maximal synergistic effect was investigated.

MATERIALS AND METHODS

Materials Human recombinant TNF-α (rhTNF), produced in *Escherichia coli* and purified (99.9%),[18] was generously provided by Asahi Chemical Industrial Co., Ltd. (Tokyo). The TNF used had a specific activity of 2.3×10^6 U/mg protein as determined from its cytotoxicity toward L-M cells,[19] and a molecular mass of 51,000 as a trimer. The *p*-nitrophenyl phosphoric acid disodium salt, *p*-nitrophenyl-β-D-glucuronide and 2,2′-bipyridine were purchased from Sigma (St. Louis, MO), while dimethyl sulfoxide (DMSO) was provided by Wako Pure Chemical Industries, Ltd. (Osaka).

Cell culture L-M (mouse tumorigenic fibroblast, ATCC CCL 137) cells, which are widely accepted as standard cells for the cytotoxicity assay of TNF, were obtained from the American Type Culture Collection (Rockville, MD). They were cultured in Eagle's minimal essential medium (Nissui Pharmaceutical Co. Ltd., Tokyo) with 10% heat-inactivated fetal bovine serum (FBS, Flow Laboratories, Inc., North Ryde, Australia), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in an incubator with 5% CO_2.

***In vitro* assessment of cytotoxicity** L-M cells (1×10^4 cells/200 μl) were added to each well of a 96-well microculture plate (Sumitomo Bakelite Co. Ltd., Tokyo), and incubated at 37°C for 4 h in 5% CO_2. After aspiration of the medium, rhTNF ($0.02–2\times10^3$ U/200 μl) was added and incubated at various temperatures for the periods specified in Fig. 1. Cytotoxicity was assessed in terms of dye uptake, which was directly proportional to cell number, as described previously.[17]

Assay of lysosomal enzyme activity Cells (1×10^6/ml) were lysed by pipetting in a hypotonic buffer (0.25 *M* sucrose/1 m*M* EDTA/10 m*M* acetic acid/10 m*M* triethanolamine, pH 7.4) by the method of Miskimins and Shimizu.[20] The resulting solution was centrifuged at 800*g* for 10 min to obtain the supernatant.

The total acid phosphatase and β-glucuronidase activities were determined by modifications of the method of Shibko and Tappel[21] and that of Kato *et al.*,[22] respectively. An aliquot of the supernatant (100 μl) was incubated for 90 min with 200 μl of 0.5 *M* sodium acetate buffer (pH 5.0 for acid phosphatase, pH 4.0 for β-glucuronidase), 100 μl of 0.2% Triton X-100, and 100 μl of 100 m*M* substrate (*p*-nitrophenyl phosphate for acid phosphatase, *p*-nitrophenyl-β-glucuronide for β-glucuronidase).

The reaction was stopped by adding 500 μl of 1% (w/v) phosphotungstic acid in 0.1 *N* HCl. The mixture was then centrifuged at 10,000*g* for 10 min to remove the precipitated protein. After the addition of 200 μl of 1 *N* NaOH to 800 μl of the resulting supernatant, the release of *p*-nitrophenol was measured using a Hitachi spectrophotometer (Model U-3200, Tokyo) at a wavelength of 405 nm. Results were expressed as milliunits (mU) per 1×10^6 cells or a percentage versus the respective controls. One unit refers to the amount of enzyme required to release 1 μmol (μ*M*) of the product per min.

Quantification of hydroxyl radical formation The production of hydroxyl radical was measured in terms of the formation of methane from DMSO in accordance with the method of Repine *et al.*[23] Each 2-ml reaction mixture was prepared in a siliconized 3.5-ml glass tube (Pierce, Rockford, IL) by the sequential addition of 1 ml of cell suspension (2×10^7/ml), and 0.8 ml of DMSO solution (2 *M*). The tube was then sealed with a siliconized Teflon septum and open-top screw cap, and 0.2 ml of the same 20 m*M* HEPES medium containing rhTNF (100 U/ml) was injected through the septum. This mixture was mixed vigorously and incubated for 1 h at 37°C or 42°C and then for 17 h at 37°C in a shaking water bath. The reaction was terminated by placing the tube on melted ice.

A 0.2-ml sample of the headspace gas from each tube was withdrawn into a gas-tight syringe (Hamilton, Reno, NV), after mixing vigorously by depressing and withdrawing of the plunger at least 10 times. The concentration of methane in the 0.2-ml gas sample was determined on a Shimadzu GC-9A gas chromatograph (Shimadzu, Tokyo) equipped with a flame-ionization detector and a 3-mm×2-m stainless steel column packed with 60/80 mesh active carbon (Gasukuro Kogyo, Tokyo) as described previously.[16]

RESULTS

Effects of hyperthermia on the cytotoxic action of rhTNF The cytotoxic effects on L-M cells of rhTNF in conjunction with the elevation of incubation temperature from 37 to 40 and 42°C appear in Table I. When the relationship between duration of TNF contact and its cytotoxic effect was determined at 37°C, the ID_{50} for TNF was

Table I. Influence of Hyperthermia on 50% Inhibitory Dose of rhTNF against L-M Cells

Temperature	ID_{50} (U/ml)[a] incubation time with TNF		
	2 h	4 h	8 h
37°C	11,000	4,000	2,300
40°C	6,000	820	680
42°C	54	4	< 1

a) Cell survival was measured by dye uptake assay following the incubation of L-M cells with 0 to 10,000 U/ml of rhTNF for 2 h, 4 h and 8 h and then in rhTNF-free medium at 37°C for 46 h, 44 h and 40 h. Heat treatment (40°C or 42°C) was done in the middle of each incubation period with rhTNF.

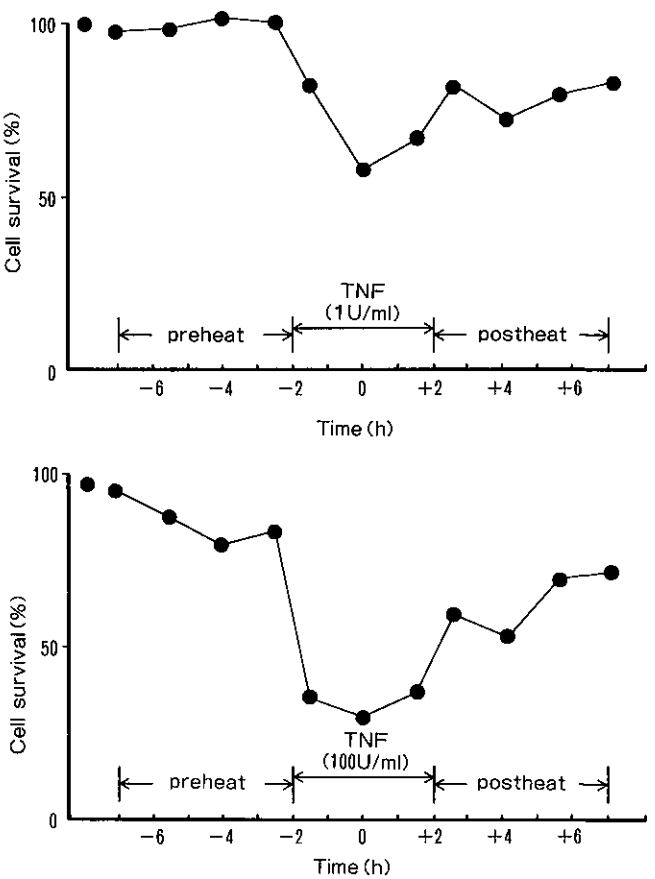

Fig. 1. Effects of timing and sequence of administration of rhTNF and hyperthermia. At various times before or after rhTNF exposure: 1 U/ml (upper panel), or 100 U/ml (lower panel) for 4 h. L-M cells were heated at 42°C for 1 h. Cells were incubated for 44 h after rhTNF exposure and cell survival was determined by dye uptake assay. Data represent cell survival at each point of 1 h heating. Survival at only heat treatment appears on the left side of each graph.

11,000 U/ml after 2 h of treatment, while at 4 and 8 h of treatment the ID_{50} values were 4,000 U/ml and 2,300 U/ml, respectively. The cytotoxic effect of TNF was dependen on the duration of its contact with tumor cells and the ID_{50} was reduced to about 1/5 of the value at 2 h, after 8 h of treatment. This tendency was also observed with combined TNF and hyperthermia at 40°C and 42°C. At 40°C the ID_{50} was reduced to about 1/9, and at 42°C to below 1/54. Hyperthermia had such a pronounced effect on the cytotoxic effect of TNF that after 2 h of contact with TNF at 40°C and 42°C the ID_{50} was reduced to about 1/18 and 1/204 the value obtained at 37°C. Similar reductions in ID_{50} were observed after 4 or 8 h of contact.

Timing of TNF and hyperthermia in combination Before and after 4 h of treating the L-M cells with TNF (1,100 U/ml), hyperthermia at 42°C was applied for 1 h, and its effect on the cytotoxic action of TNF was studied to determine the optimal timing of TNF and hyperthermia in combination (Fig. 1). The cytotoxic effect of TNF on L-M cells was determined after 4 h of treatment with TNF and 44 h of incubation of TNF-free cultures.

When rhTNF and hyperthermia were combined, a maximal cytotoxic effect was obtained; cell survival rates were 57.6% and 28.9%, when 1 U/ml and 100 U/ml of TNF, respectively, were added to the cultures.

On the other hand, when L-M cells were heated before the exposure to TNF, the cytotoxic effect was virtually unchanged. When hyperthermia was applied after the exposure to TNF, the cytotoxic effect was enhanced slightly. The synergistic effect of TNF and hyperthermia in combination decreased with increase in the interval between the TNF treatment and administration of hyperthermia.

Effect of hyperthermia on lysosomal enzyme activity The activities of acid phosphatase and β-glucuronidase in the L-M cells were determined at 37°C, 40°C, 42°C and 43°C, and at 0. 30, 60 and 90 min to study the effect of hyperthermia on lysosomal enzyme activities (Fig. 2). After 90 min of exposure to TNF at 43°C the activities of

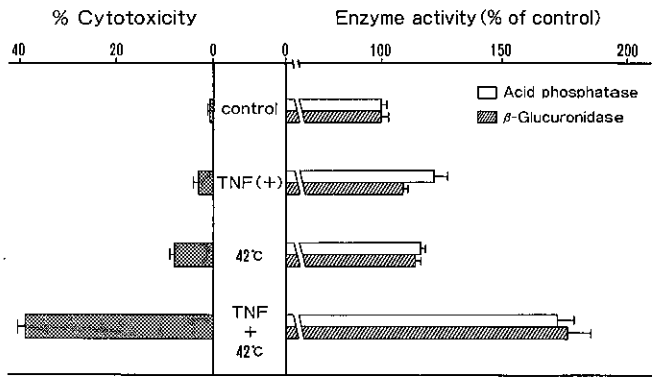

Fig. 3. Effects of hyperthermia on rhTNF susceptibility and lysosomal enzyme activity of L-M cells. Cells were incubated with or without rhTNF (100 U/ml) for 4 h. Heat treatment (42°C) was administered for the first hour. After 4 h culture, % cytotoxicity and enzyme activity were measured by the method described in "Materials and Methods." Values are the mean ± SD of three separate experiments.

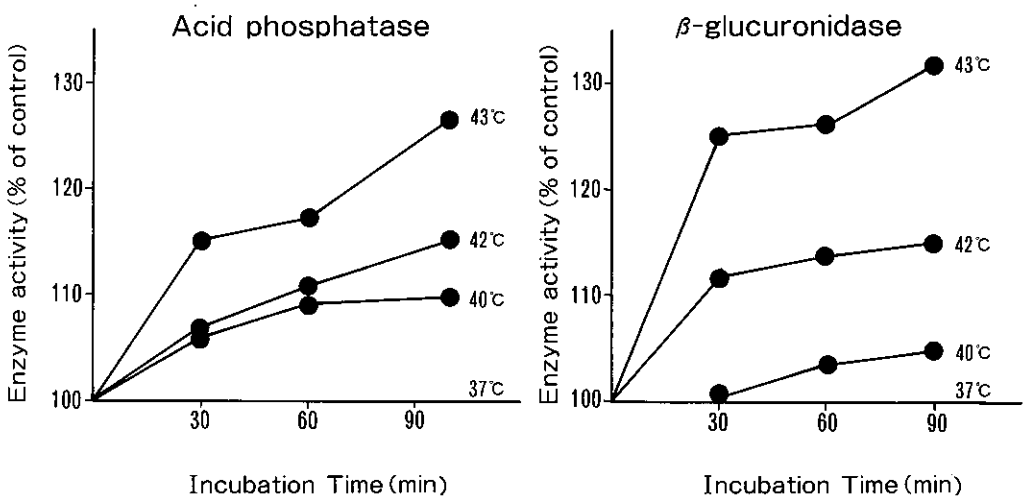

Fig. 2. Effects of duration of hyperthermia on lysosomal enzyme activity of L-M cells. Cells were incubated at 37°C, 40°C, 42°C and 43°C for 30 min, 60 min and 90 min. Enzyme activity was measured as described in "Materials and Methods." Values are the mean of three separate experiments.

Table II. Effects of Reactive Oxygen Scavengers on Susceptibility of L-M Cells to rhTNF

	Agent	% Cytotoxicity	Relative sensivity to TNF
37°C	None	46.8±1.10	1.00
	DMSO	12.1±1.70	0.26
	Bipyridine	40.9±1.60	0.87
42°C	None	65.3±0.64	1.40
	DMSO	24.1±2.50	0.51
	Bipyridine	48.7±3.20	1.04

Cells were cultured in the presence of rhTNF (100 U/ml) and reactive oxygen scavengers for 24 h treated with or without hyperthermia (42°C, 1 h). Values are the mean±SD of three separate experiments.

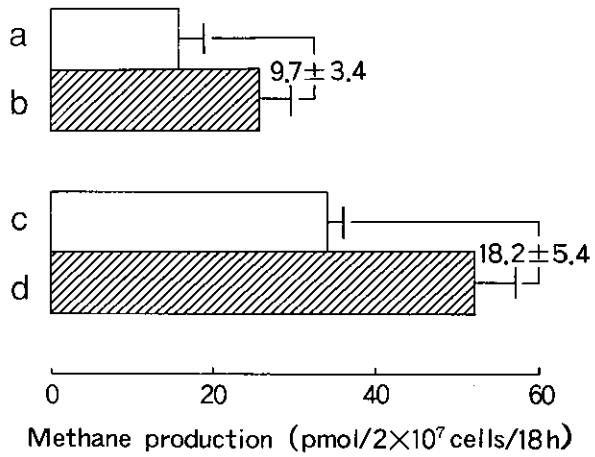

Fig. 4. Effects of hyperthermia on the production of methane by L-M cells incubated with rhTNF. L-M cells (2×10^7 cells/2 ml) were incubated for 18 h at 37°C (a, b) or 42°C (c, d) in medium containing 400 m*M* DMSO with (b, d) or without (a, c) rhTNF (100 U/ml). The methane produced was assessed by gas chromatography as described in "Materials and Methods." Values are mean±SD of three separate experiments.

acid phosphatase and β-glucuronidase were increased by 1.26 and 1.32 times as compared to values obtained at 37°C.

Effect of TNF and hyperthermia in combination on lysosomal enzyme activity The change in lysosomal enzyme activity in the L-M cells exposed to both TNF and hyperthermia was determined to evaluate the relationship of such enzyme activity to the cytotoxic effect of TNF (Fig. 3). L-M cells were cultured for 4 h with the addition of 100 U/ml of TNF, and heated to 42°C for 1 h. The cells were lysed immediately after incubation and intracellular acid phosphatase and β-glucuronidase activities were determined. Whereas these enzymes showed a 1.1- to 1.2-fold increase in activity after exposure to either TNF or heat, after a combination of TNF and hyperthermia both enzymes showed a significant (1.7-fold) increase. When L-M cells were treated under the same conditions, the cytotoxicities were 3.1% and 8.2%, respectively, following treatment with either TNF or heat alone; a synergistic cytotoxic effect of 39.2% was observed with TNF and hyperthermia in combination.

Influence of hydroxyl radical scavenger and iron chelator on the synergistic effect of TNF and hyperthermia DMSO, a hydroxyl radical scavenger, and bipyridine, an iron chelator that inhibits hydroxyl radical production, were added to L-M cell cultures to determine their influence on the synergistic effect of TNF and hyperthermia in combination (Table II). With the concurrent addition of DMSO (400 m*M*) or bipyridine (60 μM), the cytotoxic effect of TNF (100 U/ml) was inhibited to 26% or 87% of that obtained without DMSO or bipyridine, even at the control temperature of 37°C. After 1 h of hyperthermia at 42°C, on the other hand, the cytotoxic effect of TNF was enhanced 1.4-fold as compared to the value obtained at 37°C. This synergistic effect was also inhibited by the addition of DMSO or bipyridine. These results suggest that the production of hydroxyl radical is involved in the synergistic cytotoxic effect of TNF and hyperthermia in combination.

Effect of hyperthermia on hydroxyl radical production in the TNF-stimulated L-M cells The effect of hyperthermia on hydroxyl radical production in the TNF-stimulated L-M cells was determined (Fig. 4). The production of methane by L-M cells treated with TNF (100 U/ml) for 18 h (Fig. 4b) was 25.7 pmol/2×10^7 cells, a significant increase compared to 16.0 pmol/2×10^7 cells by untreated L-M cells (Fig. 4a). When the L-M cells received only heat treatment (42°C) for 1 h (Fig. 4c), methane production was increased about 2.1-fold to 34.3 pmol/2×10^7 cells as compared to the value obtained at 37°C (Fig. 4a). After combined treatment with TNF and hyperthermia (Fig. 4d), there was a pronounced increase in the production of methane to 52.5 pmol/2×10^7 cells, in excess of the combined output following separate treatments with TNF and heat (i.e., sum total of b-a and c-a).

DISCUSSION

Signal transduction is believed to occur in the tumor cell when TNF is bound to the cell surface receptor, giving rise to an increase in hydroxyl radical production and the activation of lysosomal enzyme.[13-17,24] Such a chain reaction is thought to be responsible in part for the autolysis of tumor cells exposed to TNF. Furthermore, it is known that the toxic effect of TNF on tumor cells is temperature-dependent.[11-13,25,26] We previously demon-

strated a synergistic antitumor effect of TNF and hyperthermia both *in vitro* and *in vivo* in mice subcutaneously transplanted with Meth-A cells[11] and in pulmonary metastasis models.[12]

However, the mechanism of the synergistic effect of the combination of TNF and hyperthermia is not completely clear. We have thus far shown the following: (1) the turnover of TNF is increased when TNF is combined with hyperthermia,[12] and (2) the lethal effect of hyperthermia and TNF[27] on tumor cells has cell-cycle specificity, their effect being increased in the M & S and G_2-M phases, respectively. We suggest a complementary effect of TNF and hyperthermia on the cell-phase response.

Thus, the intracellular events evoked by the combination of TNF and hyperthermia needed clarification. Accordingly, in the present study we determined the combined effect of TNF and hyperthermia on lysosomal enzyme activity and on hydroxyl radical production in the L-M cells.

It had been reported that elevated activity of lysosomal enzyme such as acid phosphatase and β-glucuronidase in tumor cells subjected to hyperthermia is associated with its cytotoxic effect.[28] This study confirmed that the production of hydroxyl radical is increased in tumor cells exposed to hyperthermia. Our findings and results suggest that the lethal effect of hyperthermia may be partially accounted for (as with TNF), by the assumption that the hydroxyl radical activates the lysosomal enzyme by the induction of lipid peroxidation in lysosomal membrane,[29] and also directly causes fragmentation of the DNA chain.[30] We showed in this study that TNF or hyperthermia induces similar intracellular events. More importantly, the combination of TNF and hyperthermia produced not only a synergistic cytotoxic effect but also a synergistic increase in lysosomal enzyme activities and hydroxyl radical production in the tumor cells as compared to when TNF and hyperthermia are evaluated separately.

The mechanism of the synergistic effect of TNF and hyperthermia in combination is of particular interest. The timing of the combination was varied to determine the influence on the cytotoxic effect. Th greatest enhancement occurred with the simultaneous administration of TNF and hyperthermia, with a slight enhancement observed when TNF stimulation was followed by hyperthermia at short intervals. However enhancement was not noted, when heat-treated L-M cells were subsequently exposed to TNF.

Hyperthermia may enhance the cytotoxic effect of activated lysosomal enzymes and increase the production of hydroxyl radicals resulting from the signal transduction mediated by the TNF receptor. Our results suggest that TNF and hyperthermia must be administered concurrently for a maximal synergistic antitumor effect.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

(Received November 16, 1991/Accepted February 5, 1992)

REFERENCES

1) Carswell, E. A., Old, L. J., Russel, R. L., Green, M. and Williamson, B. An endotoxin induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA*, **72**, 3600–3670 (1975).
2) Matthews, N. Tumor necrosis factor from the rabbit. II. Production by monocytes. *Br. J. Cancer*, **38**, 310–315 (1978).
3) Niitsu, Y., Watanabe, N., Sone, H., Neda, H., Yamauchi, N. and Urushizaki, I. Mechanism of the cytotoxic effect of tumor necrosis factor. *Jpn. J. Cancer Res.*, **76**, 1193–1197 (1985).
4) Helson, L., Green, S., Carswell, E. A. and Old, L. J. Effect of tumor necrosis factor on cultured human melanoma cells. *Nature*, **285**, 791–792 (1975).
5) Matthews, N. and Watkins, J. F. Tumor necrosis factor from the rabbit. I. Mode of action, specificity and physiochemical properties. *Br. J. Cancer*, **38**, 302–319 (1978).
6) Ruff, M. R. and Gifford, G. E. Tumor necrosis factor. *In* "Lymphokines," ed. E. Pick, pp. 235–302 (1981). Academic Press, Inc., New York.
7) Watanabe, N., Niitsu, Y., Neda, H., Sone, H., Yamauchi, N., Umetsu, T. and Urushizaki, I. Antitumor effect of tumor necrosis factor against various primarily cultured human cancer cells. *Jpn. J. Cancer Res.*, **76**, 1115–1119 (1985).
8) Watanabe, N., Niitsu, Y., Umeno, H., Kuriyama, H., Neda, H., Yamauchi, N., Maeda, M. and Urushizaki, I. Toxic effect of tumor necrosis factor on tumor vasculature in mice. *Cancer Res.*, **48**, 2179–2183 (1988).
9) Niitsu, Y., Watanabe, N., Neda, H., Yamauchi, N., Maeda, M., Sone, H. and Kuriyama, H. Induction of synthesis of tumor necrosis factor in human and murine cell lines by exogenous recombinant human tumor necrosis factor. *Cancer Res.*, **48**, 5407–5410 (1988).
10) Himeno, T., Watanabe, N., Yamauchi, N., Maeda, M., Tsuji, Y., Okamoto, T., Neda, H. and Niitsu, Y. Expression of endogenous tumor necrosis factor as a protective

protein against the cytotoxicity of exogenous tumor necrosis factor. *Cancer Res.*, **50**, 3941–4945 (1990).
11) Watanabe, N., Niitsu, Y., Umeno, H., Sone, H., Neda, H., Yamauchi, N., Maeda, M. and Urushizaki, I. Synergistic cytotoxic and anti-tumor effects of recombinant human tumor necrosis factor and hyperthermia. *Cancer Res.*, **48**, 650–653 (1988).
12) Niitsu, Y., Watanabe, N., Umeno, H., Sone, H., Yamauchi, N., Maeda, M. and Urushizaki, I. Synergistic effects of recombinant human tumor necrosis factor and hyperthermia on *in vitro* cytotoxicity and artificial metastasis. *Cancer Res.*, **48**, 654–657 (1988).
13) Kull, F. C., Jr. and Cuatrecasas, P. Possible requirement of internalization in the mechanism of *in vitro* cytotoxicity in tumor necrosis serum. *Cancer Res.*, **41**, 4885–4890 (1981).
14) Watanabe, N., Neda, H., Ohtsuka, Y., Sone, H., Yamauchi, N., Maeda, M., Kuriyama, H. and Niitsu, Y. Signalling pathway of tumor necrosis factor in normal and tumor cells. *Cancer Immunol. Immunother.*, **28**, 157–163 (1989).
15) Liddil, J. D., Dorr, R. T. and Scuderi, P. Association of lysosomal activity with sensitivity and resistance to tumor necrosis factor in murine L929 cells. *Cancer Res.*, **49**, 2722–2728 (1989).
16) Yamauchi, N., Kuriyama, H., Watanabe, N., Neda, H., Maeda, M. and Niitsu, Y. Intercellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells *in vitro*. Cancer Res., **49**, 1671–1675 (1989).
17) Yamauchi, N., Watanabe, N., Kuriyama, H., Neda, H., Maeda, M., Himeno, T., Tsuji, Y. and Niitsu, Y. Suppressive effects of intracellular glutathione on hydroxyl radical production induced by tumor necrosis factor. *Int. J. Cancer*, **46**, 884–888 (1990).
18) Shirai, T., Yamaguchi, H., Ito, H., Toda, C. W. and Wallace, R. B. Cloning and expression in *Escherichia coli* of the gene for human tumor necrosis factor. *Nature*, **313**, 803–806 (1985).
19) Yamazaki, N., Onishi, E., Enami, K., Natori, K., Kohase, N., Sakamoto, H., Tanouchi, M. and Hayashi, H. Proposal of standardized methods and reference for assaying recombinant human tumor necrosis factor. *Jpn. J. Med. Sci. Biol.*, **39**, 105–118 (1986).
20) Miskimins, W. K. and Shimizu, N. Dual pathway for epidermal growth factor processing after receptor-mediated endocytosis. *J. Cell. Physiol.*, **112**, 327–338 (1982).
21) Shibko, S. and Tappel, A. L. Rat-kidney lysosomes: isolation and properties. *Biochem. J.*, **95**, 731–741 (1965).
22) Kato, K., Yoshida, K., Tsukamoto, H., Nobunaga, M., Masuya, T. and Sawada, T. Synthesis of p-nitrophenyl β-D-glucopyranosiduronic acid and its utilization as a substrate for the assay of β-glucuronidase activity. *Chem. Pharm. Bull.*, **8**, 239–242 (1960).
23) Repine, J. E., Ealon, J. V., Anders, N. W., Hoidal, J. R. and Fox, R. B. Generation of hydroxyl radical by enzymes, chemicals and human phagocytes *in vitro*: detection with the anti-inflammatory agent, dimethyl sulfoxide. *J. Clin. Invest.*, **64**, 1642–1651 (1979).
24) Watanabe, N., Kuriyama, H., Sone, H., Neda, H., Yamauchi, N., Maeda, M. and Niitsu, Y. Continuous internalization of tumor necrosis factor receptors in a human myosarcoma cell line. *J. Biol. Chem.*, **263**, 10262–10266 (1988).
25) Ruff, M. R. and Gifford, G. E. Rabbit tumor necrosis factor: mechanism of action. *Infect. Immun.*, **31**, 380–385 (1981).
26) Tsujimoto, M., Yip, Y. K. and Vilcek, J. Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. *Proc. Natl. Acad. Sci. USA*, **82**, 7626–7630 (1985).
27) Watanabe, N., Niitsu, Y., Yamauchi, N., Neda, H., Sone, H., Maeda, M. and Urushizaki, I. Cell cycle specificity of tumor necrosis factor and its receptor. *Cell Biol. Int. Rep.*, **11**, 813–817 (1987).
28) Keech, M. L. and Wills, E. D. The effect of hyperthermia on activation of lysosomal enzymes in HeLa cells. *Eur. J. Cancer*, **5**, 1025–1031 (1979).
29) Tong-Mak, I., Misra, H. P. and Weglicki, W. B. Temporal relationship of free radical-induced lipid peroxidation and loss of latent enzyme activity in highly enriched hepatic lysosomes. *J. Biol. Chem.*, **258**, 13733–13737 (1983).
30) Sugioka, K., Nakano, M., Tero-Kubota, S. and Ikegami, Y. Generation of hydroxyl radicals during the enzymatic reductions of the Fe^{3+} ADP-EDTA systems. *Biochim. Biophys. Acta*, **753**, 411–421 (1983).