Synergistic Effects of Recombinant Human Tumor Necrosis Factor and Hyperthermia on in Vitro Cytotoxicity and Artificial Metastasis

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ABSTRACT

Synergy in cytotoxic effect between recombinant human tumor necrosis factor and hyperthermia (incubation at 38.5°C or 40°C) was observed to occur against L-M (mouse tumorigenic fibroblast) cells and shown to be related to an accelerated turnover rate of recombinant human tumor necrosis factor-receptor complex under elevated temperatures rather than to changes in number of cell receptors or binding strength. However, no synergy in cytotoxic effect was observed to occur against human embryonic lung (HEL) cells.

A clearly synergistic inhibition of metastatic tumor growth by combined administration of recombinant human tumor necrosis factor (300 units) and whole-body hyperthermia (40°C, 30 min) was also observed in BALB/c mice previously given injections of 1 × 10^6 Meth-A (MH) cells/mouse via tail vein, neither of which alone resulted in significant inhibition.

INTRODUCTION

TNF is an anticancer monokine with demonstrated in vitro cytotoxicity against cultured cell lines (1-4) and tumor cells from cancer patients (5) and inhibition of transplanted tumor growth (1, 6) and lung metastasis (7) in vivo. Its cytotoxic spectrum is somewhat limited (5), however, and attempts have been made to overcome this disadvantage for clinical applications and to find combinations with various anticancer agents (6, 8) or interferon (6, 9, 10) which will provide more potent therapeutic effects.

The cytotoxic action of TNF on cancer cells is known to vary with temperature (11-13). The present study was undertaken to investigate the mechanism of this synergy and its effect on pulmonary metastasis of Meth-A cells following their i.v. injection in mice.

MATERIALS AND METHODS

Cell Culture. L-M (mouse tumorigenic fibroblast) cells were cultured in Eagle MEM (Nissui) containing 5% FCS (Flow Laboratories); HEL cells in Eagle MEM containing 10% FCS; and Meth-A (MH) cells characterized by a high disposition to metastasis to the lungs, a variant of Meth-A (mouse fibrosarcoma) cells, in RPMI 1640 containing 10% FCS (GIBCO). All cultures were incubated in a humidified, 5% CO₂ atmosphere at 37°C. L-M cells and HEL cells were kindly supplied by the Department of Pathology, Sapporo Medical College. Meth-A cells were provided by Dr. S. Sato, Department of Internal Medicine (Section 4), Sapporo Medical College.

rH-TNF. rH-TNF, produced in Escherichia coli (14) and purified (99.9%), was generously provided by Asahi Chemical Industry Co. The preparation had a specific activity of 2.3 × 10⁶ units/ml and was contained in <0.1 mg of endotoxin/ml as measured by colorimetric Limulus amebocyte lysate assay kit (Pyrodick; Seikagaku Kogyo Co., Tokyo, Japan).

In Vitro Assay for Cytotoxic Action. Portions of rH-TNF (0.1-10⁴ units/ml) and L-M cells or HEL cells (1 × 10⁴ cells/ml) were added to the wells of a microculture plate (Sumitomo Bakelite), and the plate was incubated at 37, 38.5, or 40°C for 1, 6, or 12 h. The cells were further incubated in TNF-free Eagle MEM containing 10% FCS or RPMI 1640 containing 10% FCS at 37°C in 5% CO₂ for 47, 42, or 36 h, for a total incubation time of 48 h. The cytotoxic activity was then assessed by the dye uptake method (15).

Binding Assay. Recombinant human TNF was labeled with ¹²⁵I by the method of Bolton and Hunter (16). The ¹²⁵I-labeled rH-TNF had a specific activity of 2.6 × 10⁵ cpm/µg. After incubation of L-M cells at 37°C or 38.5°C under 5% CO₂ for 30 min, 6 h, or 12 h, the supernatant was washed out and rH-TNF binding to the L-M cells was assayed by incubating the pretreated L-M cells in tissue culture culture cluster (Costar) wells at 4 × 10⁵ cells/well at 1 h at 4°C with 5 nM ¹²⁵I-rH-TNF and 3.7 µM carrier rH-TNF, washing the cells five times with Eagle MEM containing 0.1% FCS, solubilizing the radioactivity associated with the cell surface with 0.5 N sodium hydroxide solution, and performing measurements in a gamma counter.

The number of rH-TNF receptors per cell and the dissociation constant (Kₐ) were determined by Scatchard plot analysis.

Analysis of Internalization and Degradation of rH-TNF. L-M cells were placed in tissue culture cluster (Costar) wells at 5 × 10⁵ cells/well and incubated for 1 h at 4°C with 5 nM ¹²⁵I-TNF. After pulse labeling, the plate was incubated at 37°C or 38.5°C for 5, 10, or 20 min, followed by counting of radioactivity in trypsin released (bound to cell surface receptor), trypsin released (internalized), and TCA-soluble supernatant (degradation product) fractions (17).

Assessment of Activity against Artificial Lung Metastasis. Meth-A (MH) cells, 5 × 10⁵ in 50 µl RPMI 1640, were injected into the caudal vein of female BALB/c mice (6 weeks old; Clea Japan, Inc.), and 300 units of rH-TNF or 0.1 ml of saline were injected at the same site after 2 days (Experiment 1) or 2 and 4 days (Experiment 2). The treatment was immediately followed by hyperthermia; animals were anesthetized by i.p. injection of secobarbital sodium (24 mg/kg), and then fixed in 50-ml plastic centrifuge tubes (Falcon) having 16 holes each 7 mm in diameter. The tubes were then lowered into a water bath at 40°C to immerse the mouse body. Rectal and intratracheal temperatures were measured with a thermometer (TM-54; Inter Nova Cor.) and confirmed to reach approximately the same temperature as the water bath within 5 min after initial immersion.

The lungs were removed 14 days after the tumor cell injection, and the pulmonary surface nodules were counted.

RESULTS

Cytotoxicity in Vitro. The effect of temperature on rH-TNF cytotoxicity was assessed on the basis of the ID₉₀ under incubation for 1, 6, and 12 h (Table 1). With L-M cells, which are highly susceptible to TNF cytotoxicity, the elevated incubation temperatures had relatively little effect on the ID₉₀ values at 1 h, but at 6 and 12 h they resulted in a marked decrease in ID₉₀ values, indicating a potentiation of rH-TNF cytotoxicity by heating. The decreases in ID₉₀ under 12 h of incubation at 38.5 and 40°C corresponded, respectively, to 125- and 500-fold increases over the cytotoxicity observed with incubation at 37°C.

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1 The abbreviations used are: TNF, tumor necrosis factor; Eagle MEM, Eagle's minimal essential medium; FCS, fetal calf serum; HEL, human embryonic lung; rH-TNF, recombinant human tumor necrosis factor; TCA, trichloroacetic acid; ID₉₀, rH-TNF dose necessary for 50% cytotoxicity.
Table 1  Influence of hyperthermia on 50% inhibitory dose* against L-M cells

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Units/ml at following combination time</th>
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<tbody>
<tr>
<td></td>
<td>1 h</td>
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<tr>
<td>37°C</td>
<td>460</td>
</tr>
<tr>
<td>38.5°C</td>
<td>400</td>
</tr>
<tr>
<td>40°C</td>
<td>360</td>
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</table>

* Cell survival was measured by dye uptake assay after incubation of L-M cells with 0 to 10,000 units/ml of rH-TNF at 37°C, 38.5°C, and 40°C for 1 h, 6 h, and 12 h and then in TNF-free medium at 37°C for 42 h, 42 h, and 36 h. Each value was corrected for cell survival determined in the absence of rH-TNF at 37°C, 38.5°C, or 40°C for 1, 6, or 12 h.

Fig. 1. Influence of hyperthermia on rH-TNF cytotoxicity against HEL cells. Determination of cell survival by dye uptake assay incubation of HEL cells with 0 to 10,000 units/ml of rH-TNF at 37°C, 38.5°C, and 40°C for 6 h (left) and 12 h (right) and then in TNF-free medium at 37°C for 42 h (left) and 36 h (right).

Table 2  TNF receptors on the surface of L-M cells pretreated with hyperthermia

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>No./cell $\times 10^5$</th>
<th>$K_d$ (x $10^{-10}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>8.8</td>
<td>5.7</td>
</tr>
<tr>
<td>6 h</td>
<td>8.9</td>
<td>5.5</td>
</tr>
<tr>
<td>12 h</td>
<td>8.7</td>
<td>5.6</td>
</tr>
<tr>
<td>38.5°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>8.9</td>
<td>5.7</td>
</tr>
<tr>
<td>6 h</td>
<td>8.3</td>
<td>5.4</td>
</tr>
<tr>
<td>12 h</td>
<td>8.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>

L-M cells were preincubated for 30 min, 6 h, or 12 h at 37°C and 38.5°C. After washing, cells were incubated for 1 h with 125I-labeled TNF (5 ntu) at 4°C. The number of TNF receptors and affinity of TNF binding to its receptor ($K_d$) were then determined as described in "Materials and Methods."

Fig. 2. Time course of binding (left), internalization (middle), and degradation (right) of 125I-labeled rH-TNF to L-M cells at 37°C and 38.5°C. L-M cells (6 x $10^5$ cells/well) were incubated with 5 ntu 125I-labeled TNF at 37°C (●) and 38.5°C (□) for the indicated times. Cell-associated radioactivity not released by treatment with trypsin was measured as described in "Materials and Methods." Radioactivity soluble in 10% TCA was also measured in the culture medium. Bars, SE.

HEL cells, as normal diploid cells, are generally not susceptible to TNF cytotoxicity. Although their susceptibility appeared to increase slightly under incubation with rH-TNF at 38.5°C and 40°C, the ID$_{50}$ values remained well in excess of 10,000 units of rH-TNF (Fig. 1).

The heat sensitivity of HEL cells in vitro was almost the same as that of L-M cells (data were not shown).

Cell Binding, Internalization, and Degradation. The numbers of TNF receptors on the L-M cells after incubation without rH-TNF at 37°C and 38.5°C for 30 min, 6 h, or 12 h, as determined by Scatchard plot analysis, were, respectively, 8.7-8.9 x 10$^3$ and 8.3-8.9 x 10$^3$ cells (Table 2). The dissociation constant for the binding was on the order of 10$^{-10}$ M in both cases. Thus, thermal treatment appeared to have no significant effect on either receptor number or binding strength.

Incubation of L-M cells with 125I-labeled rH-TNF at 37°C and 38.5°C showed the higher temperature to have no significant effect on cell binding or internalization of rH-TNF. However, the radioactivity of the TCA-soluble (degradation product) fraction after incubation at 38.5°C for 5 and 10 min was significantly higher than at 37°C ($P < 0.05$), as shown in Fig. 2.

Effect of Artificial Lung Metastasis. Under the protocol shown in Fig. 3, the administration of rH-TNF at 300 units and/or hyperthermia at 40°C was performed on the 2nd day or on the 2nd and 4th days after i.v. injection of Meth-A (MH) cells into the BALB/c mice, when micrometastatic formation had already occurred in the lung (Fig. 4). Autopsy of all animals was performed on the 14th day after tumor cell injection to determine the incidence of lung metastasis and the number of pulmonary surface nodules. As shown in Table 3, pulmonary metastasis was observed in all of the mice.

The number of pulmonary surface nodules in each of the two groups receiving rH-TNF alone was similar to that in the mice of the respective control group, which received only saline injections: 188.4 ± 17.1 (SE) in the group receiving rH-TNF on day 2 only as against 153.0 ± 10.8. The mean number was somewhat lower in each of the groups receiving only hyperthermia, but not to a statistically significant degree.

In each of the two groups receiving rH-TNF and hyperthermia in combination, the mean number of pulmonary nodules was significantly lower. In the groups receiving both on day 2, it was 106 ± 4.4, which would indicate an inhibition of 42.5 and 43.7% against the control and TNF alone groups, respec-
SYNERGY BETWEEN TNF AND HYPERTHERMIA

Fig. 4. Histology of lung 2 days after i.v. injection of Meth-A (MH) cells showing micrometastasis (arrow). H & E, × 100.

Table 3  Synergistic effect of TNF and hyperthermia on pulmonary metastasis formed by i.v. injection of Meth-A (MH) cells

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Combination therapy</th>
<th>Incidence of pulmonary metastasis</th>
<th>No. of pulmonary surface nodules (mean ± SE)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>7/7</td>
<td>180 ± 15.7</td>
<td>100</td>
</tr>
<tr>
<td>Hyperthermia</td>
<td>Day 2</td>
<td>7/7</td>
<td>140.2 ± 12.7</td>
<td>76.0</td>
</tr>
<tr>
<td>TNF, 300 units</td>
<td>Day 2</td>
<td>7/7</td>
<td>188.4 ± 17.1</td>
<td>102.1</td>
</tr>
<tr>
<td>TNF, 300 units + hyperthermia</td>
<td>Day 2</td>
<td>7/7</td>
<td>106.0 ± 4.4</td>
<td>57.5</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Control</td>
<td>9/9</td>
<td>153.0 ± 10.8</td>
<td>100</td>
</tr>
<tr>
<td>Hyperthermia</td>
<td>Days 2 and 4</td>
<td>8/8</td>
<td>129.1 ± 12.9</td>
<td>84.4</td>
</tr>
<tr>
<td>TNF, 300 units</td>
<td>Days 2 and 4</td>
<td>9/9</td>
<td>157.5 ± 11.8</td>
<td>102.9</td>
</tr>
<tr>
<td>TNF, 300 units + hyperthermia</td>
<td>Days 2 and 4</td>
<td>8/8</td>
<td>68.3 ± 9.3</td>
<td>44.6</td>
</tr>
</tbody>
</table>

* TNF was administered i.v. 4 days after injection of Meth-A (MH) cells (5 × 10⁶) into the caudal vein of BALB/c mice. The treatment was immediately followed by as described in "Materials and Methods." Metastasis was measured on day 14.

n.s., not significant.

The cytotoxic effect of rH-TNF is known to vary with temperature (11–13). The present study was undertaken to characterize the mechanism of this synergism in vitro and to investigate its effect on artificially induced pulmonary metastasis in mice.

Incubation with rH-TNF at 38.5°C and 40°C for 12 h resulted in a cytotoxic effect on L-M cells which was 125 and over 500 times higher, respectively, that at 37°C but had little or no effect on the susceptibility of normal diploid HEL cells to rH-TNF.

Incubation at 38.5°C had no significant effect on the number of rH-TNF receptors on L-M cells or their rate of rH-TNF internalization, but the amount of rH-TNF degradation product (as observed in TCA-soluble fraction of supernatant) in the L-M cells after 5 and 10 min at 38.5°C was significantly higher than that at 37°C. The results thus suggest that acceleration of rH-TNF metabolism by heating may play one of the roles in the synergism.

Various studies have recently demonstrated that hyperthermia can markedly improve the local therapeutic effects of chemotherapy (18–20) or radiation therapy (20–23). Investigation has generally been limited to effects on superficial tumors, however, and animal tests on the effects of combination therapy with hyperthermia on deep-body tumors are necessary before its testing in clinical trials.

The results of the present study, with pulmonary metastasis induced in mice by i.v. injection of Meth-A (MH) cells, indicate that hyperthermia can markedly enhance the growth inhibition effect of rH-TNF on tumors deep within the body. Neither rH-TNF at 300 units/ml nor hyperthermia at 40°C had any appreciable therapeutic effect, but in combination they clearly inhibited metastatic tumor development. This was particularly apparent in the mice which received combination therapy twice, 2 and 4 days after injection of tumor cells, as the number of pulmonary surface nodules was significantly lower than that in mice treated with hyperthermia alone, as well as that of the controls.

**DISCUSSION**

The cytotoxic effect of rH-TNF is known to vary with temperature (11–13). The present study was undertaken to characterize the mechanism of this synergism in vitro and to investigate its effect on artificially induced pulmonary metastasis in mice.

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Incubation at 38.5°C had no significant effect on the number of rH-TNF receptors on L-M cells or their rate of rH-TNF internalization, but the amount of rH-TNF degradation product (as observed in TCA-soluble fraction of supernatant) in the L-M cells after 5 and 10 min at 38.5°C was significantly higher than that at 37°C. The results thus suggest that acceleration of rH-TNF metabolism by heating may play one of the roles in the synergism.

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The results suggest that hyperthermia can be used in combination with rH-TNF to elicit a more potent anticancer effect from rH-TNF in deep-body as well as superficial tumors.

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