Synergistic Cytotoxic and Antitumor Effects of Recombinant Human Tumor Necrosis Factor and Hyperthermia

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ABSTRACT

A synergistic increase in the cytotoxic effects of recombinant human tumor necrosis factor (rH-TNF) and hyperthermia was demonstrated both in vitro and in vivo. The cytotoxicity of rH-TNF against L-M cells in incubation for 12 h at 38.5 °C was based on the concentration necessary for 50% cytotoxicity was, respectively, 125 and more than 500 times as high as in similar incubation at 37°C. As observed 18 days after implantation of Meth-A fibrosarcoma cells in mice, single i.v. administration of rH-TNF at 1000 units/mouse resulted in complete cures in five mice when performed in combination with hyperthermia (40°C), whereas rH-TNF alone in the same dose resulted in 27.1% inhibition of tumor growth and hyperthermia alone had no appreciable effect on tumor growth. The i.v. administration of rH-TNF three times at 100 or 300 units/mouse together with hyperthermia (40°C) resulted in 41.2 and 89.0% tumor growth inhibition, respectively; similar administration without hyperthermia appeared to have little or no appreciable effect on tumor growth. The results suggest that combination therapy including rH-TNF and hyperthermia may be of value in the treatment of malignancy in human patients.

INTRODUCTION

The cytotoxic activity of rH-TNF has been confirmed in experiments with various cell lines (1-4) and tumor cells obtained from cancer patients (5). In mice bearing tumor transplants, rH-TNF has been shown to inhibit pulmonary metastasis (6).

Administration of rH-TNF to patients with leukemia has reportedly resulted in a marked reduction in the number of peripheral leukemia cells (7). However, the cytotoxic spectrum of rH-TNF is somewhat limited. Susceptibility to rH-TNF varies even among tumor cells of similar tissue origin (5). Effective combination therapies are therefore desirable to overcome differences in susceptibility to TNF, improve its therapeutic effect, and ameliorate its side effects. In the present study, we therefore examined the cytotoxic and antitumor effects of rH-TNF in combination with hyperthermia.

MATERIALS AND METHODS

Cell Lines. L-M and Meth-A cells were kindly provided by Asahi Chemical Industry Co., Ltd. and Dr. S. Sato, Department of Pathology, Sapporo Medical College. L-M cells were maintained in Eagle's MEM containing 10% fetal calf serum (Flow Laboratories) in a humidified 5% CO2 atmosphere at 37°C. Meth-A cells were passaged i.p. administration of rH-TNF at 1000 units/mouse resulted in complete cures in five mice when performed in combination with hyperthermia (40°C), whereas rH-TNF alone in the same dose resulted in 27.1% inhibition of tumor growth and hyperthermia alone had no appreciable effect on tumor growth. The i.v. administration of rH-TNF three times at 100 or 300 units/mouse together with hyperthermia (40°C) resulted in 41.2 and 89.0% tumor growth inhibition, respectively; similar administration without hyperthermia appeared to have little or no appreciable effect on tumor growth. The results suggest that combination therapy including rH-TNF and hyperthermia may be of value in the treatment of malignancy in human patients.

Materials and methods

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In Vitro Assessment of Cytotoxicity. Portions of 1 to 1 x 104 units/ml rH-TNF (Asahi Chemical Industry Co., Ltd.) (8) and a 1 x 107/ml suspension of L-M cells, which are widely accepted as standard cells for cytotoxic assay of TNF (1, 3, 5, 9), were added to the wells of a 96-well microculture plate and incubated at 37, 38.5, or 40°C for 1, 6, or 12 h in 5% CO2. The TNF solution and cell suspension were adjusted for each temperature with preheated medium before incubation.

The cells were then rinsed three times with TNF-free Eagle's MEM containing 10% fetal calf serum and further incubated in the same medium for 47, 42, or 36 h at 37°C in 5% CO2 for a total incubation time of 48 h. Cytotoxicity was then assessed by the dye uptake method, which was directly proportional to cell number, as described previously (9).

Meth-A Cell Implantation in Mice. L-M cells were not used for the in vivo experiments, since they did not form detectable tumors when they were implanted s.c. Instead, Meth-A cells, which are generally used for the assay of in vivo antitumor effects of TNF, were implanted s.c. although they were somewhat less sensitive to the in vitro cytotoxicity of TNF as compared to L-M cells (1). The Meth-A cell in vitro has almost the same heat sensitivity as that of the L-M cell (data not shown). Female BALB/c mice were purchased from Clea Japan, Inc. and maintained in a specific pathogen-free room at 25 ± 2°C. Mice aged 6 weeks (body weight, 23 g) were submitted to the experiments. A cell suspension of 1 x 106 cells/ml was prepared in Eagle's MEM, and 0.1 ml of the cell suspension (1 x 106 cells) was implanted s.c. in the abdominal aspect of BALB/c mice.

Administration of rH-TNF. rH-TNF was injected into the caudal vein in a single dose of 1000 units 6 days after implantation of the tumor cells or in three doses of 100 or 300 units each 6, 10, and 14 days after the implantation. The tumors were 6 to 8 mm in diameter on the sixth day after implantation.

Hyperthermia. Tumor-bearing mice were anesthetized by i.p. injection of secobarbital sodium (24 mg/kg), administered rH-TNF or 0.9% NaCl solution i.v. via tail vein, 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 so as to immerse the whole mouse body including the tumor bearing portion.

Rectal and intratumoral temperatures were measured with a thermistor thermometer (TM-54; Inter Nova Corp.), and it was confirmed that they reached approximately the same temperature as that of the water bath within 5 min after initial immersion (Fig. 1).

In Vivo Evaluation of Antitumor Effects. Tumor growth curves were

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Fig. 1. Intratumor and rectal temperatures during initial period of whole-body hyperthermia of BALB/c mice bearing Meth-A fibrosarcoma transplanted s.c. at 1 x 106 cells/mouse by immersion in a water bath at 40°C for 30 min on the sixth day after transplantation. Temperature readings were made with thermistor thermometers (TM-54; Inter Nova Corp.) at 3 mm depth in tumor (C) and 2 cm from anus (O).
growth inhibition of 5.6 and 20.6%, respectively. On the 11th day, 4-5 days before the appearance of spontaneous necrosis as to the extent of necrosis over the tumor surface: ++++, necrosis of entire surface area; +++, necrosis of more than one-half of surface area; +, necrosis of less than one-half of surface area; —, no necrosis observed on surface.

RESULTS

Cytotoxicity in Vitro. The cytotoxic effects on L-M cells of rH-TNF in conjunction with the elevation of incubation temperature from 37 to 38.5 and 40.0°C are shown in Fig. 2. When the tumor cells were incubated in the absence of rH-TNF, the number of viable cells in the culture at 38.5°C was not significantly different from that at 37°C for up to 12 h of incubation. After incubation at 40°C for 1, 6, and 12 h, the mean number of viable cells was lower than the corresponding number at 37°C by 8.1, 22.0, and 34.8%, respectively.

Under incubation at 37°C with rH-TNF in low concentrations (1 to 10 units/ml), the cytotoxicity increased with increasing incubation time. Nevertheless, the concentration of rH-TNF necessary for 50% cytotoxicity remained constant, at about 300 units/ml, regardless of the duration of incubation for up to 12 h.

The mean number of viable tumor cells present after 1 h of incubation with rH-TNF at 38.5 and 40.0°C was not significantly different from that after 1 h at 37.0°C. After 6 and 12 h, however, the cytotoxic effect at the higher temperatures was markedly stronger; in comparison with the 50% toxicity values after 6 and 12 h at 37°C, those at 38.5°C were, respectively, 2.3 and 125 times smaller and those at 40.0°C were, respectively, 119.4 and over 500 times smaller.

Antitumor Effects. As shown in Figs. 3 and 4, the ratio between the mean size of tumors in the control group (saline, 0.1 ml) on the 18th day after transplantation and that on the 6th day (day of first injection) was 5.52. In the groups receiving rH-TNF alone in injections of 100 or 300 units/mouse on 3 days, it was, respectively, 5.21 and 4.38, thus indicating a mean growth inhibition of 5.6 and 20.6%, respectively. On the 11th day, 4-5 days before the appearance of spontaneous necrosis as part of the natural growth cycle, partial tumor necrosis (+ or ++) was observed in 16.6% (1 of 6) of the mice receiving 100-unit injections and 66.7% (4 of 6) of those receiving 300-unit injections (Table 1). In the group receiving hyperthermia (40°C) alone, the ratio between the mean tumor sizes on the 18th and 6th days was 5.48 (Figs. 3 and 4), indicating little or no inhibition of tumor growth.

The mean tumor size ratios for the groups receiving both hyperthermia and rH-TNF in three injections of 100 or 300 units/mouse were, respectively, 3.34 and 1.75, indicating growth inhibitions of 39.4 and 68.3% (Figs. 3 and 4). On the 11th day, 24 h after the second rH-TNF injection, partial necrosis (+, ++) was observed in 57.1 (4 of 7) and 100% (6 of 6), respectively (Table 1).

In the group receiving rH-TNF only in one injection of 1000 units/mouse, partial tumor necrosis (+, ++) was observed in 90% (9 of 10) 24 h after injection, i.e., the seventh day after transplanting (Table 2).

In the control group (saline, 0.1 ml), the ratio of mean tumor sizes on the 18th and 6th days after transplanting was 6.27 (Fig. 5). In the group receiving rH-TNF only (1000 units/mouse), it was 4.26, indicating a growth inhibition of 32.2%. In the group receiving hyperthermia (40°C) alone, it was 5.32, an inhibition of only 15.2% (Fig. 5). In the group receiving both rH-TNF (1000 units/mouse) and hyperthermia (40°C), the ratio between mean tumor sizes on the 15th and 6th days was 0.8, indicating a 95.6% growth inhibition, and disappearance of tumors was observed in all cases (5 of 5) on the 18th day (Fig. 5 and Table 2).
SYNERGISTIC EFFECT OF TNF AND HYPERTERMIA

DISCUSSION

TNF is a monokine which holds strong promise for application in cancer therapy because of its marked antitumor effects (1, 6, 7). Human recombinant TNF (8, 11–13) has been developed and is currently the subject of phases I and II clinical trials. Studies are being done on the possibility of its combined use with other therapeutic methods or agents in order to increase its antitumor activity in preparation for clinical applications in the near future. Some of these studies have used γ-interferon (14–16) and various other anticancer agents (7, 17) to enhance the antitumor effect of rH-TNF.

The cytotoxic activity of TNF has been known to vary depending on temperature (18–20). In the present study, as shown in Fig. 1, the susceptibility of tumor cells to rH-TNF during incubation at 38.5 and 40°C for 12 h was about 125 and 500 times as high, respectively, as that at 37°C. In the experiments in vivo, the combined use of hyperthermia with three doses of 100 or 300 units of rH-TNF inhibited the growth of tumors by

Table 2 Effect of rH-TNF and hyperthermia in combination on necrotic response and cure rate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 7</th>
<th>Day 11</th>
<th>Day 15</th>
<th>Complete cure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>Saline + hyperthermia</td>
<td>6.5</td>
<td>3.5</td>
<td>3.5</td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>TNF</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>TNF + hyperthermia</td>
<td>6.5</td>
<td>3.5</td>
<td>3.5</td>
<td>0/10 (0)</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of single rH-TNF injection of 1000 units and hyperthermia in combination. Administration of rH-TNF at 1000 units/mouse on sixth day following transplantation was done with or without hyperthermia at 40°C for 30 min; all other conditions are as in Fig. 3. Plots, mean ± SE (bars); *, P < 0.05, **, P < 0.01 by Student's t test; arrow, the period of rH-TNF administration and hyperthermia.
SYNERGISTIC EFFECT OF TNF AND HYPERTHERMIA

41.2 and 69.0%, respectively, as assessed 18 days after the transplantation; the same dosage of rH-TNF alone appeared to have had little effect on tumor growth at the end of the same period.

A single injection of rH-TNF at 1000 units/mouse without hyperthermia resulted in the appearance of tumor necrosis in 9 of 10 mice bearing Meth-A tumors and a mean tumor growth inhibition of 27.1% as measured on the 18th day after transplanting. Under the same conditions, no significant antitumor effect was observed after treatment with hyperthermia alone. With the combined treatment of rH-TNF at 1000 units/mouse and hyperthermia at 40°C, however, the mean growth inhibition was 95.6% on the 15th day and disappearance of tumors was observed on the 18th day in all five mice.

The results suggest that combination therapy including rH-TNF and hyperthermia may be of value in the treatment of malignancy in human patients for increased antitumor effect.

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REFERENCES
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