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Enhanced Antitumor Effect of Recombinant Human Tumor Necrosis Factor in Combination with Recombinant Human Granulocyte Colony-stimulating Factor in BALB/c Mice

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The synergistic antitumor effect of tumor necrosis factor (TNF) and granulocyte colony-stimulating factor (G-CSF) was investigated. G-CSF was administered subcutaneously to BALB/c mice inoculated with Meth-A cells at a dose of 2.5 μg/day for 5 consecutive days. When TNF (1×10^3 U) was administered intravenously to mice which had been pretreated with G-CSF, tumor growth showed a 74.1% inhibition 17 days after the tumor cell inoculation, compared to that of untreated mice. In this experiment, G-CSF significantly ($P<0.025$) enhanced the antitumor effect of TNF. The *in vitro* cytotoxicity of TNF (10 U/ml) towards Meth-A cells was increased about 5.2-fold in the presence of neutrophils (E/T=50) as compared to the cytotoxicity obtained with TNF alone. A combination of TNF and G-CSF (50 ng/ml) in the presence of neutrophils, resulted in a 2.1 times greater cytotoxicity against Meth-A cells as compared to that obtained without G-CSF. Significant augmenting effects of G-CSF on superoxide (O_2^-) production by TNF-stimulated neutrophils were observed. These observation suggest that the neutrophil plays an important role in the antitumor action of TNF on Meth-A cells, and that the antitumor effect of TNF is enhanced by combination with G-CSF.

Key words: Tumor necrosis factor — Granulocyte colony-stimulating factor — Antitumor effect — Meth-A cells

Granulocyte colony-stimulating factor (G-CSF) has been reported to activate neutrophils by promoting the differentiation of hematopoietic stem cells into mature neutrophils, stimulating the proliferation of mature neutrophils, prolonging their life,[1-3] and enhancing their production of superoxide (O_2^-).[4-8] It has also been found that G-CSF not only enhances the antibody-dependent cell-mediated cytotoxicity (ADCC) of neutrophils against tumors,[9] but also promotes their production of interferon-α.[10] These actions of G-CSF have received considerable attention, and its clinical application in cancer therapy has been investigated.

Like G-CSF, tumor necrosis factor (TNF), an antitumor cytokine, activates neutrophils *in vitro*.[11, 12] The TNF-stimulated neutrophils exhibit a cytotoxic effect against various cancer cells in culture.[13] While it is anticipated that the neutrophil plays an important function in the *in vivo* antitumor effect of TNF, no direct evidence has yet been reported.

Intrigued by these suggested actions of G-CSF, we designed a study to determine whether the antitumor effects of TNF would be enhanced in Meth-A cell-inoculated Balb/c mice having a G-CSF-induced elevation in neutrophil count. We attempted to clarify the mechanism of the synergistic effect of TNF and G-CSF by conducting an *in vitro* analysis of the influence of neutrophils and G-CSF on the cytotoxicity of TNF against Meth-A cells. Furthermore, the augmenting effect of G-CSF on O_2^- production by TNF-stimulated neutrophils was examined.

MATERIALS AND METHODS

Animals 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 used in the experiments.

Materials Recombinant human TNF,[14] which was produced in *Escherichia coli* and then purified, was generously provided by Asahi Chemical Industry Co., Tokyo. This preparation had a specific activity of 2.3×10^6 U/mg protein, in terms of its cytotoxic activity against mouse L-M cells, and contained <0.1 ng of endotoxin/mg protein as determined by a colorimetric assay (*Limulus* amebocyte lysate assay kit, Pyrodick; Seikagaku Kogyo Co., Tokyo).

Recombinant human G-CSF,[1, 15] which was produced in *E. coli* and then purified, was generously provided by Kirin Brewery Co., Tokyo. This preparation had a specific activity of 1×10^8 U/mg protein as determined in an assay of granulocyte colony-forming units.

Cell lines Meth-A cells, which were kindly provided by Dr. N. Sato, Department of Pathology, Sapporo Medical

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College, were passaged intraperitoneally in BALB/c mice.[16]

Administration of G-CSF Six days after the implantation of Meth-A cells, G-CSF was injected subcutaneously at a daily dose of 2.5 μg/mouse for 5 consecutive days. Leukocytes and neutrophils were counted by means of a Hematology Analyzer (Sysmex F-800, Toa Medical Electronics Co., Ltd., Kobe).

Administration of TNF and *in vivo* evaluation of anti-tumor effects TNF was injected into the caudal vein as a single dose of 1×10^3 or 3×10^3 U, given the day after the first injection of G-CSF (Fig. 1). Tumor growth curves were then plotted from the estimated tumor mass (mg), calculated from the formula $V=ab^2/2$, where a is the maximum lengthwise diameter of the tumor, and b is the minimum diameter perpendicular to a, as measured with calipers.[16]

Preparation of neutrophils Neutrophil preparations were obtained from healthy adult donors by the dextran sedimentation and Ficoll-Isopaque methods.[17]

***In vitro* assessment of cytotoxicity** Meth-A cells (2×10^6) were labeled with ^{51}Cr by incubation for 2 h at 37°C with 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston) in 1 ml of RPMI 1640 (Gibco, New York) with 10% fetal bovine serum (FBS, Flow Laboratories, Australia). Tumor cells were washed three times before being used as target cells. ^{51}Cr-Labeled Meth-A cells (1×10^4/well) were added to the wells of a 24-well microculture plate (Falcon^R 3047) with or without neutrophils (5×10^5/well). TNF (10 U/ml) and/or G-CSF (50 ng/ml) were added followed by incubation in 5% CO_2 at 37°C for 48 h. The percent cytotoxicity was calculated from the formula $100(a-b)/(c-b)$, where a, b and c represent the counts/min of experimental release, spontaneous release and maximum release (obtained by adding Nonidet P-40 1% in distilled water), respectively.[18, 19]

Neutrophil O_2^- release assay The O_2^- production was measured in terms of by superoxide dismutase (SOD) inhibitable reduction of cytochrome c, using the modified microtechnique of Pick and Mizel.[20] Briefly, cells were suspended in flat-bottomed, 96-well microtiter tissue culture plates (Falcon^R 3072) at a concentration of 1×10^6/100 μl in Hanks' balanced salt solution without phenol red, pH 7.2 (Nissui Pharmaceutical Co., Tokyo), containing cytochrome c acid modified from horse heart (Sigma Chemical Co., St. Louis), 1 mg/ml. TNF was used as the stimulus at a concentration of 10 U/ml with or without G-CSF at a concentration of 50 ng/ml. The plates were incubated for various lengths of time at 37°C under 5% CO_2 and read in a EASY READER^R EAR 340 AT (SLT Labinstruments) at 550 nm. Five replicate wells were employed for each determination. Wells to which 300 U/ml SOD from bovine erythrocytes (Sigma Chemical Co.) had been added served as blanks. Results were expressed in nmoles $O_2^-/10^6$ cells, using an extinction coefficient of 21×10^3 $M^{-1}cm^{-1}$, corrected for the calculated length of the light path.

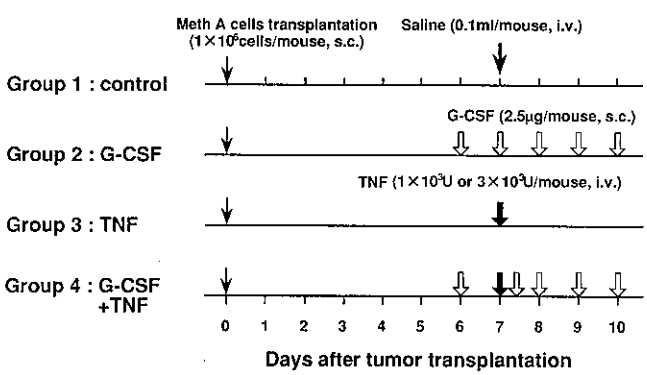

Fig. 1. Study design.

RESULTS

Effects of G-CSF on leukocyte (neutrophil) count To determine when G-CSF would be most effectively combined with TNF, G-CSF was injected subcutaneously

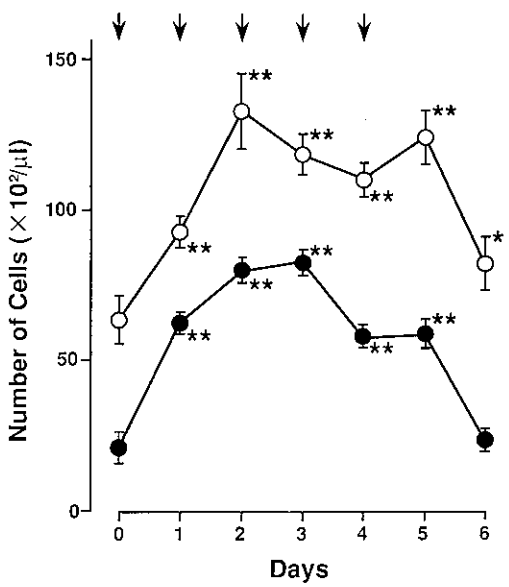

Fig. 2. Effect of G-CSF on total number of leukocytes and neutrophils of BALB/c mice. G-CSF was injected subcutaneously at a total daily dose of 2.5 μg/mouse for 5 consecutive days. Leukocytes (○) and neutrophils (●) were counted by means of a hematology analyzer. Values, mean±SD (bars) of 5 mice; *, $P<0.05$, **, $P<0.01$ by Student's t test; arrow, the period of G-CSF injection.

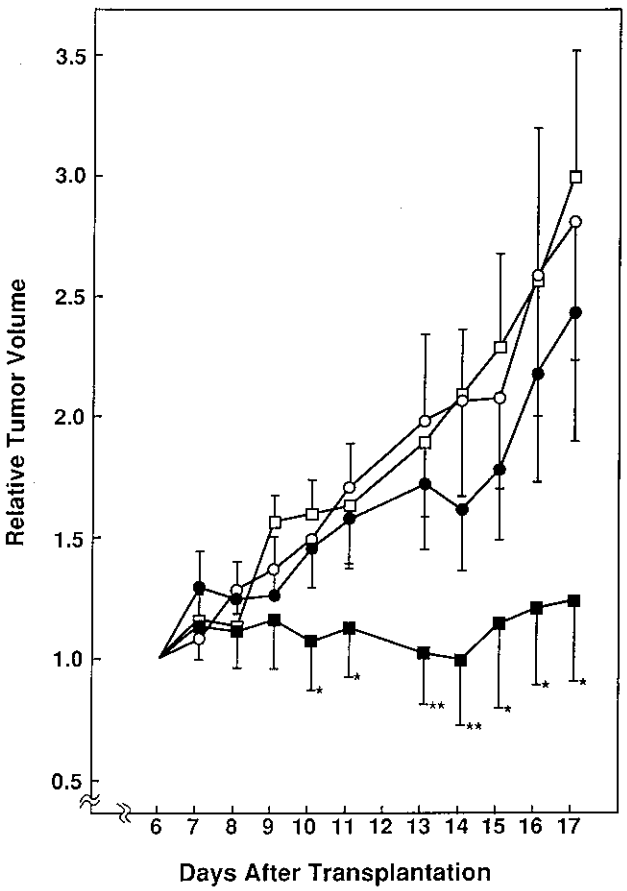

Fig. 3. Effect of G-CSF and TNF (1×10^3 U) in combination on tumor growth in BALB/c mice. ○, saline; ●, TNF only; □, G-CSF only; ■, TNF and G-CSF. Study design is as in Fig. 1. Values, Mean±SE (bars) of 7 mice; *, $P<0.05$, **, $P<0.025$ by Student's t test.

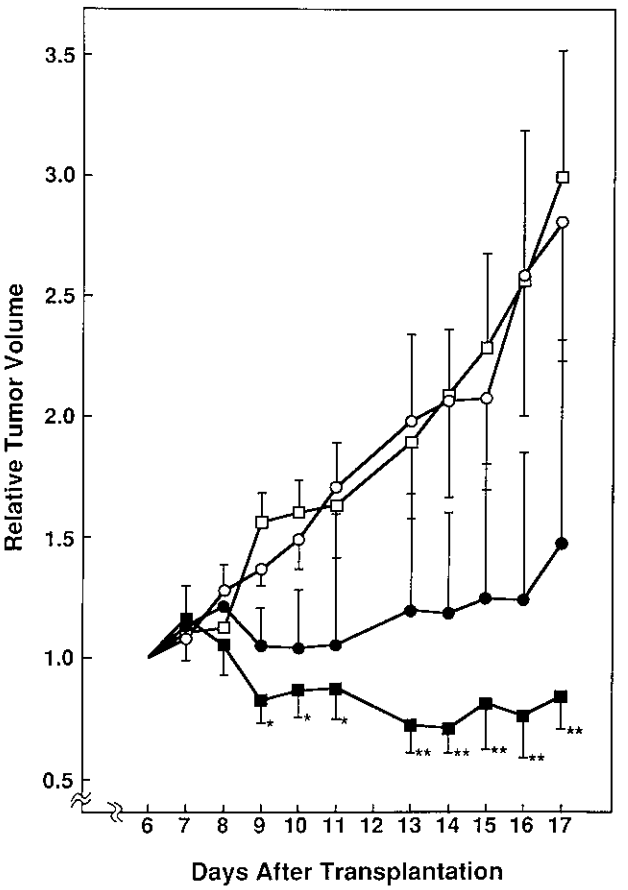

Fig. 4. Effect of G-CSF and TNF (3×10^3 U) in combination on tumor growth in BALB/c mice. ○, saline; ●, TNF only; □, G-CSF only; ■, TNF and G-CSF. Study design is as in Fig. 1. Values, mean±SE (bars) of 7 mice; *, $P<0.05$, **, $P<0.025$ by Student's t test.

into the abdominal cavity of BALB/c mice at a daily dose of 2.5 μg for 5 consecutive days. The changes in peripheral leukocyte and neutrophil counts were then determined at specified intervals post-therapy (Fig. 2).

Two days after the initiation of G-CSF, the leukocyte count had almost doubled and the neutrophil count had almost quadrupled. Thereafter both counts reached a plateau, returning to pretherapy values 2 days after the completion of G-CSF administration.

Effects of G-CSF on antitumor activity of TNF The optimal time for the administration of TNF was determined to be the day after the initiation of G-CSF, when the neutrophil count was significantly increased ($P<0.01$) compared to that of control mice given saline (Fig. 2). The effect of G-CSF on the antitumor activity of TNF was determined in BALB/c mice inoculated with Meth-A cells (Figs. 3 and 4). There was no significant difference in tumor growth between the group receiving G-CSF vs. the control group given saline. The group that received TNF alone at a dose of 1×10^3 U showed no difference in tumor growth vs. the control group. The group that received TNF at a dose of 3×10^3 U showed a 49.0% inhibition of tumor growth 17 days after the inoculation of tumor cells. TNF alone exhibited no appreciable antitumor effect at a daily dose of 1×10^3 U but when this dose was administered in combination with G-CSF, it produced a 74.1% inhibition of tumor growth 17 days after the inoculation of tumor cells (Fig. 3).

The antitumor activity of TNF given at a dose of 3×10^3 U was increased by 21% when combined with G-CSF as compared to the effect of TNF given alone 17 days after the inoculation of tumor cells (Fig. 4).

Effects of neutrophils and G-CSF on TNF cytotoxicity against Meth-A cells We used a dose of 10 U/ml of

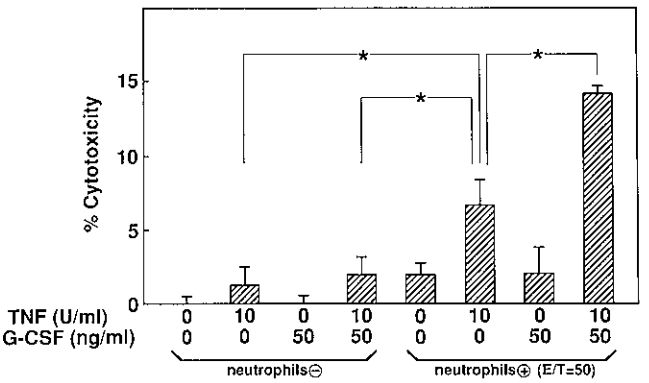

Fig. 5. Influence of G-CSF and human neutrophils on the cytotoxicity of TNF against Meth-A cells. Values are mean ± SD of three separate experiments. *, $P<0.001$ by Student's t test.

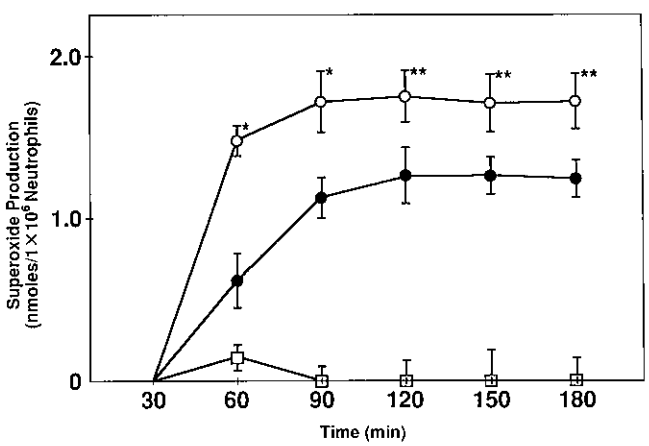

Fig. 6. Effects of G-CSF on O_2^- release by TNF-stimulated neutrophils. Values are mean ± SD of five separate experiments. Neutrophils (1×10^6/100 μl) were incubated with TNF (●); G-CSF (□); TNF and G-CSF (○). O_2^- release was measured as SOD-inhibitable reduction of cytochrome c, as described in "Materials and Methods." *, **, values significantly (*, $P<0.001$, **, $P<0.002$) different from values without G-CSF.

TNF for *in vitro* experiments, which approximates to the serum concentration of mice receiving 1×10^3 or 3×10^3 U of TNF intravenously.[21] The dose of G-CSF was determined to 50 ng/ml based on the result that the optimal priming effect of G-CSF on O_2^- production was obtained at 25–50 ng/ml.[7]

The cytotoxicity (%) of either TNF (10 U/ml) or neutrophils given alone against Meth-A cells after 48 h of culture was about 1.3% or 2.0%, respectively. G-CSF alone showed no cytotoxic activity against the Meth-A cells, and the cytotoxicity of TNF was not enhanced by the addition of G-CSF (Fig. 5). However, in the presence of neutrophils (E/T=50), the TNF cytotoxicity was increased by about 5.2 times to 6.7%, as compared with the value obtained in the absence of neutrophils. The addition of G-CSF, further increased the percent cytotoxicity by 2.1 times to 14.3%, as compared with the value obtained without added G-CSF. These findings suggest that the cytotoxic activity of the TNF-stimulated neutrophils is enhanced in the presence of G-CSF.

Effects of G-CSF on O_2^- production by TNF-stimulated neutrophils The time course of O_2^- production in TNF (10 U/ml)-stimulated neutrophils with or without G-CSF (50 ng/ml) is shown in Fig. 6. In the absence of G-CSF, the cumulative O_2^- production increased during the first 120 min of incubation, then apparently reached a plateau. The O_2^- production in the presence of G-CSF in a similar incubation was significantly higher than that without G-CSF throughout the entire investigated incubation time (up to 180 min).

DISCUSSION

Besides acting at the level of the myelopoietic stem cells and precursor cells in promoting the differentiation and proliferation of neutrophils, G-CSF mobilizes mature neutrophils from the bone marrow pool, prolongs their life,[1-3] and enhances their function.[4-8, 22] Thus, until recently the clinical application of G-CSF was directed primarily at the prevention of infection in patients with leukocytopenia induced by anticancer chemotherapy and/or radiotherapy. Recent studies, have shown that neutrophils treated with G-CSF exhibit an enhanced ADCC against the HTLV-II-infected β-lymphoblastoid cell line J-WIL-2D[9] and enhance the production of interferon (INF)-α.[10] It has also been suggested that G-CSF plays a role in the tumor supervision mechanism.[23, 24] Similarly, it has been pointed out that TNF plays a role in those mechanisms.

It has also been shown that TNF, like G-CSF, activates neutrophils *in vitro*.[11, 12] Thus, one would expect that the activation of neutrophils by TNF would have implications for the *in vivo* antitumor activity, as would its direct cytotoxic effects on tumor cells [18, 19, 25-29] and vessels[21] and its induction of antitumor macrophages[30] and activation of lymphocytes.[31] Considering these reported actions of TNF and G-CSF, we studied the effects of these agents in combination upon mice inoculated with tumor cells. Since G-CSF has no species specificity,[32] human G-CSF was used for animal (mice) experiments in the present study.

In the mice which developed an elevated neutrophil count following 5 consecutive days of subcutaneous ad-

ministration of G-CSF at a dose of 2.5 μg/mouse/day, TNF produced a 74.1% inhibition of tumor growth 17 days after the inoculation Meth-A cells, a standard cell line used in studying *in vivo* antitumor action of TNF. However, a daily dose of TNF 1×10^3 U given intravenously had no effect in the mice not exposed to G-CSF. The possibility that the antitumor activity of TNF was enhanced because the Meth-A cell is specifically sensitive to neutrophils is excluded by the finding that neutrophils stimulated by β-1, 3-D-glucan or *Corynebacterium parvum* exhibit cytotoxic activity against various tumor cells including MH134 hepatoma cells, MM48 mammary carcinoma cells,[33,34] and MOT teratoma cells.[35] Thus, our findings provide evidence to suggest that the neutrophil plays an important role in the antitumor activity of TNF, and also, that the antitumor effect of TNF is enhanced by G-CSF.

To clarify the mechanism of the synergistic effect of TNF and G-CSF given in combination, we investigated the effect of neutrophils and G-CSF on the cytotoxicity of TNF against Meth-A cells. It was shown that the cytotoxicity of TNF against these cells was enhanced in the presence of neutrophils and was further increased by the addition of G-CSF. It should be noted, however, that G-CSF alone did not exhibit cytotoxic activity against Meth-A cells, and that the cytotoxicity of TNF was not affected by the addition of G-CSF in the absence of neutrophils. These findings seem to imply that the neutrophils acquire antitumor activity when stimulated by TNF, and that their antitumor activity is further enhanced by the addition of G-CSF.

The antitumor activity of the G-CSF-stimulated neutrophils might be explicable in terms of the induction of IFN-α or of such oxygen free radicals as O_2^- and hydrogen peroxide (H_2O_2). However, no INF-α activity was detected in the supernatant of neutrophil cultures at the concentrations of G-CSF used in this study (data not shown).

It is known that when the neutrophils pretreated with G-CSF are stimulated by N-formyl-methionyl-leucyl-phenylalanine, the level of O_2^- exceeds that of untreated neutrophils.[6] Considering that there is little, if any, production of O_2^- in the neutrophils stimulated by G-CSF alone, there may be a priming effect. This idea is consistent with the observation that the G-CSF-stimulated neutrophils showed no cytotoxic activity against Meth-A cells. TNF, besides exerting a priming action, is a powerful inducer of oxygen radicals in neutrophils.[36] Thus, one may presume that the synergistic effects of G-CSF and TNF in combination in the presence of neutrophils are a consequence of the vigorous production of oxygen radicals by neutrophils that are first primed by G-CSF, then stimulated by TNF. In this study, we proved that G-CSF enhanced O_2^- production by TNF-stimulated neutrophils.

Judging from our results, the antitumor activity of TNF ought to decline with a reduction in neutrophil count. In our study, in mice whose neutrophil counts were decreased by pretreatment with an anticancer drug, the antitumor effect of TNF (3×10^4 U, i.v./mouse) was nearly completely inhibited (data not shown).

It was previously reported that TNF and anticancer drugs given in combination at the same time showed a synergistic antitumor effect.[37] Our findings suggest that TNF may, in fact, not exhibit a maximal antitumor effect in patients with a low neutrophil count induced by the administration of anticancer drugs. The antitumor activity of TNF would be utilized more efficiently in such patients if TNF were combined with G-CSF to increase the neutrophil count toward normal.

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