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Effect of Co-administration of Granulocyte Colony-stimulating Factor on Interferon Therapy

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The effect of co-administration of granulocyte colony-stimulating factor (G-CSF), as an anti-neutropenia agent, on interferon therapy was examined in a mouse model, in anticipation of an enhancement of interferon efficacy, because neutrophils induced by G-CSF are thought to act as antitumor effectors. G-CSF was intraperitoneally co-administered with human interferon α A/D (IFN) on Day 6 to Day 10 after intradermal inoculation of Meth A fibrosarcoma. Although the co-administration of G-CSF could protect against neutropenia and leukopenia induced by IFN, it did not enhance the regression of tumor, and rather reduced the prolongation of survival time and the long-term survival incidence of IFN therapy. The subsequent *in vitro* study showed that the antiproliferative activity of peripheral blood leukocytes from Meth A-bearing mice given both IFN and G-CSF was much weaker than that of mice given IFN alone. Whether the observed nullifying effect of G-CSF on IFN therapy is also the case with tumors other than Meth A is open to further study.

Key words: Interferon α A/D — Granulocyte colony-stimulating factor — Monocyte — Immunosuppression — Meth A fibrosarcoma

Our previous report showed that administration of interferon α A/D (IFN) to Meth A fibrosarcoma-bearing mice induced both tumor regression and the production of antitumor monocytes in peripheral blood of the treated mice.[1] However, the tumor was not always completely eradicated by IFN therapy. We supposed that leukopenia and neutropenia, side effects of IFN, may possibly reduce the therapeutic effect of IFN, because, as reported in several papers,[2-4] neutrophils (polymorphonuclear leukocytes) are important antitumor effector cells. Therefore, it was supposed that neutropenia induced by IFN was disadvantageous to the host from the viewpoint of tumor eradication and that an increase in the number and activation of neutrophils in peripheral blood by administration of granulocyte colony-stimulating factor (G-CSF) might enhance the antitumor reaction in the tumor-bearing host.

This study, however, failed to find enhanced therapeutic efficacy of G-CSF and IFN in combination; rather, G-CSF administration reduced the therapeutic efficacy of IFN and the antitumor activity of peripheral blood mononuclear cells of IFN-injected mice.

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MATERIALS AND METHODS

Mice and tumors Female BALB/c mice were obtained from Japan Charles River Co., Ltd. (Atsugi). Meth A fibrosarcoma in ascitic form was supplied by Dr. Y. Hashimoto.

IFN Recombinant human interferon α A/D (Bgl) was purified from *E. coli* 294 cell lysates by a combination of methods described previously.[5] The purified material was homogeneous on polyacrylamide gel electrophoresis and it had a specific activity of more than 10^8 units/mg protein as evaluated with bovine kidney cells (MDBK). Antiviral activity was titrated against the NIH standard (Gxa 01-901-535). IFN (100 μg/ml) was supplied in 0.9% NaCl solution with or without 1 mg/ml mouse serum albumin. Placebo contained the same ingredients as above except IFN, which was deleted.

G-CSF Recombinant human granulocyte colony-stimulating factor (G-CSF) (KRN8601, Kirin Amgen) purified from *E. coli*[6] was kindly provided by Kirin Brewery Co., Ltd. (Tokyo). It was shown that the purified material had a specific activity of 10^8 units/mg when assayed in a granulocyte/macrophage colony-forming unit assay.[7]

Therapy by IFN Groups of mice were inoculated intradermally (i.d.) with 2.5×10^5 or 5×10^5 tumor cells in the flank (Day 0). The mice were treated intraperi-

toneally (i.p.) with 2.5×10^5–5×10^5 units of IFN/mouse per day, 500 μg of G-CSF/kg per day, or placebo on Days 6–10. Tumor sizes were measured at the longest (a) and shortest (b) diameters and expressed as $\sqrt{ab}$. Any mouse tumor-free on Day 90 was considered as having been cured.

Differential counting of peripheral blood leukocytes (PBL) Peripheral blood for smears and counts of the total number of PBL was obtained by tail bleeding. The total PBL count (cells/mm^3) was determined in a hemocytometer by using Türk solution (Wako Pure Chemical Industries, Ltd., Osaka) which stained only nucleated cells. The differential counts of PBL were determined by using Wright-Giemsa staining.

Preparation of PBL At the indicated intervals, the mice were killed with chloroform and blood samples were obtained via heart puncture. PBL were purified from heparinized blood according to a standardized method for blood mononuclear cell preparation,[1] by Ficoll density gradient centrifugation at the density of 1.090 (M-SMF, Japan Antibody Institute, Takasaki) or 1.077 (Ficoll Paque, Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Briefly, the heparinized blood was two-fold diluted with phosphate-buffered saline, layered on M-SMF or Ficoll Paque, and then centrifuged at 1200g for 30 min. The PBL at the interface were collected and washed with Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, NY). To lyse the contaminating erythrocytes, the cells were suspended in $\times 1/5$ HBSS (as defined below) for 30 s, and then $\times 10$ HBSS (as defined below) was added to this cell suspension to adjust the osmotic pressure to the physiological value. The PBL were washed twice by centrifuging them in serum-free RPMI-1640 (GIBCO). The cell viability of the final mononuclear cell suspension was over 95% as determined by using trypan blue solution (GIBCO).

Culture medium and other reagents RPMI-1640 medium containing 100 μg kanamycin/ml (Banyu Pharmaceutical Co., Ltd., Tokyo) and either 2% BALB/c mouse serum (RPMI-M) or 10% fetal bovine serum (GIBCO) (RPMI-F) was used. Ten-fold concentrated Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} ($\times 10$ HBSS) was from GIBCO. This solution was diluted 20-fold with water (Otsuka Pharmaceutical Co., Ltd., Tokyo) and used as $\times 1/5$ HBSS.

***In vitro* antiproliferation assay** This assay was carried out according to the procedure reported previously.[1] Briefly, PBL (0.5×10^6 or 10^6/ml) were incubated with Meth A cells (0.5×10^4/ml) in RPMI-M. After three days, the surviving tumor cells were measured by regrowth assay; an aliquot of the incubation mixture was transferred into RPMI-F (1 ml) and the incubation was continued for another two days. Cell concentrations were determined by a Model Zb Coulter counter (Coulter Electronics Inc., Hialeah, FL). Cell growth of the control incubation mixture was in the middle of the logarithmic phase. Percent of control Meth A growth were calculated by using the following formula:

% of control Meth A growth = (cell counts of cultured tumor in the presence of PBL of drug-injected mice/cell counts of cultured tumor in the presence of PBL of control placebo-injected mice) $\times$ 100.

This assay gave exactly the same results as those achieved by the [^{3}H]thymidine incorporation assay, as previously reported.[1]

Statistics The results on *in vivo* incidence of long-term survival were analyzed by means of Fisher's exact test. The survival time of tumor-bearing mice was analyzed by using the Mann-Whitney U-test for the absolute counts of leukocytes and *in vitro* antiproliferation assay, Student's t test was employed.

RESULTS

Effect of G-CSF on the composition of PBL of IFN-treated Meth A-bearing mice Fig. 1 shows the kinetics of differential PBL counts of blood obtained from the tail vein of Meth A-bearing mice during the course of IFN and/or G-CSF treatment. The PBL counts on Day 11 after i.p. IFN administration were lower than those of the placebo group, but this reduction was reversed by i.p. co-injection of G-CSF.

***In vivo* effect of G-CSF on IFN therapy of Meth A-bearing mice** The therapeutic result in terms of i.d. Meth A

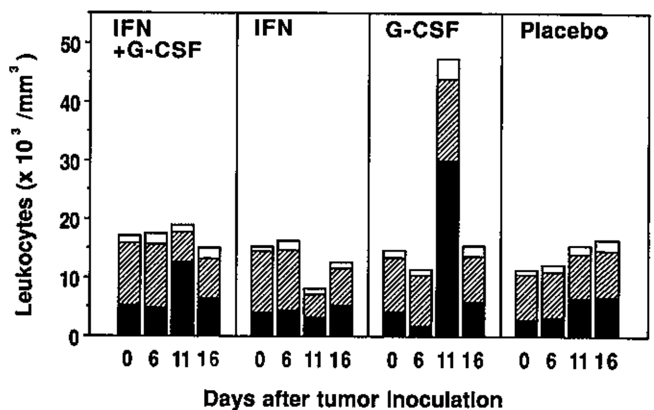

Fig. 1. Kinetics of the differential PBL counts of blood obtained from the tail vein of Meth A-bearing mice injected with 5×10^5 units of IFN, or 500 μg of G-CSF/kg or both on Days 6–10 after the tumor inoculation, compared with the placebo control. Five (IFN+G-CSF and IFN group) or four (G-CSF and placebo group) mice were used. ■, neutrophils; ▨, lymphocytes; □, other cells.

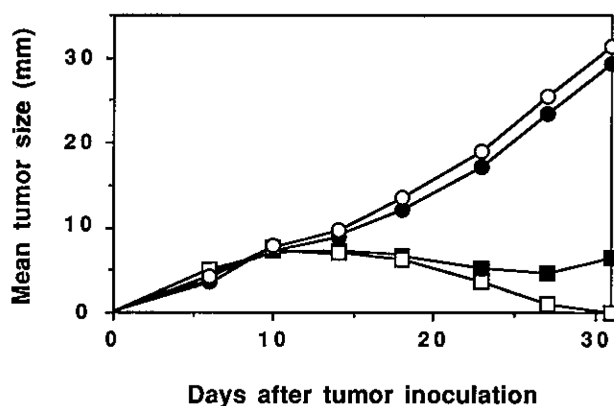

Fig. 2. Meth A growth in mice injected with IFN (□), or G-CSF (●) or both (■) or placebo (○). This figure was derived from the same experiment as that shown in Fig. 1. The long-term survival incidence in this experiment is presented in Table I, experiment 1.

Table I. Effect of G-CSF on the Outcome of IFN Treatment of Meth A-bearing Mice[a)]

Treatment	Survival of Meth A-bearing mice					
	Experiment 1		Experiment 2		Experiment 1+Experiment 2	
IFN+G-CSF	2/5[d)]	54 (20–54, >90)[e)]	1/8	48 (41–50, >90)	3/13	48 (20–54, >90)
IFN[b)]	5/5	>90 (>90)	4/8	>71.5 (43–53, >90)	9/13[f)]	>90[g)] (43–53, >90)
G-CSF[c)]	0/4	40 (34–48)	0/4	35.5 (28–38)	0/8	38 (28–48)
Placebo	0/4	36.5 (34–45)	0/5	35 (30–43)	0/9	36 (30–45)

a) Meth A cells at 5×10^5/mouse (Experiment 1) or 2.5×10^5/mouse (Experiment 2) were inoculated i.d.
b) IFN was i.p. injected at 5×10^5 (Experiment 1) or 2.5×10^5 (Experiment 2) units/mouse per day on Days 6–10.
c) G-CSF was injected i.p. at 500 μg/kg per day on Days 6–10.
d) No. of long-term survivors/total no. of mice.
e) Median survival days (range of survival days).
f) Significant at $P<0.05$ (vs. IFN+G-CSF group), 0.01 (vs. G-CSF group and placebo group) by the Fisher's exact test.
g) Significant at $P<0.05$ (vs. IFN+G-CSF group), 0.01 (vs. G-CSF group and placebo group) by the Mann-Whitney U-test.

Table II. *In vitro* Antiproliferation Activity of Peripheral Blood Leukocytes of Meth A-bearing Mice Treated with IFN and G-CSF

Treatment of mice	Ficoll gradient density[b)]	Composition of prepared PBL[a)] (%)			% Growth of Meth A cells in the presence of PBL at E/T ratio of:	
		Neutrophils	Lymphocytes	Monocytes	100	200
IFN+G-CSF	1.090	80	9	9	135±20[c)]	117±3[c)]
IFN+G-CSF	1.077	13	48	36	157±10	112±6
IFN	1.090	16	61	20	55±9[d)]	36±7[d)]
Placebo	1.090	5	78	11	100±5	100±14

Mice inoculated i.d. with 5×10^5 Meth A cells at day 0 were given 5×10^5 units IFN/mouse per day i.p. alone or in combination with 500 μg of G-CSF/kg per day or placebo on Days 6–10. On Day 11, peripheral blood leukocytes of these mice prepared by the use of the indicated separation media were incubated with Meth A at the indicated effector/target ratio for three days and the antitumor activity of these cells was determined by regrowth assay, in which aliquots of the incubation mixture were transferred and incubated in an excess of fresh medium until the surviving Meth A cells reached a sufficient number for cell counting with a Coulter counter. The results are expressed as relative percent of control in the presence of peripheral blood mononuclear cells of placebo-injected mice.
a) Peripheral blood leukocytes. Cell composition was determined by using Wright-Giemsa stain.
b) Two kinds of the Ficoll gradient to prepare PBL of mice were used; M-SMF (density, 1.090) and Ficoll Paque (density, 1.077).
c) Mean±SD of quadruplicate or triplicate determinations.
d) Statistically significant at $P<0.05$ vs. other groups tested by using Student's *t* test.

growth in Fig. 2 and the result of Fig. 1 are derived from the same experiment. The summarized results show that the co-injection of G-CSF shortened the median survival time of IFN-treated Meth A-bearing mice (Table I). The administration of G-CSF alone did not affect either the tumor growth (Fig. 2) and the survival time of Meth A-bearing mice (Table I) in this schedule. G-CSF did not greatly modify the tumor sizes at the regression stage induced by IFN (Fig. 2), but did increase the incidence of regrowth thereafter (Fig. 2, Table I), and the co-injection of IFN and G-CSF gave a lower incidence of long-term survivors (23%) than did the injection of IFN alone (69%).

Effect of co-injection of G-CSF and IFN on the *in vitro* antiproliferative activity of PBL Our previous report showed that IFN administration produced antiproliferative monocytes in the blood of Meth A-bearing mice and tumor-free normal mice and that those monocytes were associated with the therapeutic effect of IFN in the Meth A-bearing mice.[1] Thus, we examined the effect of G-CSF administration on the production of antiproliferative mononuclear cells by IFN. In preliminary experiments, many neutrophils were found to contaminate the mononuclear fraction of mice co-injected with G-CSF and IFN when separated by Ficoll density gradient centrifugation under usual conditions (M-SMF, density =1.090) for mouse lymphocytes. The contamination was greatly reduced by the use of a Ficoll density gradient (Ficoll Paque, density=1.077) for human lymphocytes. Table II shows the composition and the antitumor activity of peripheral blood leukocytes prepared with M-SMF or Ficoll Paque from mice given IFN alone or the combination of IFN and G-CSF or placebo. The PBL fraction of mice co-administered with IFN and G-CSF was composed of many neutrophils (80%) and a few mononuclear cells in the M-SMF-prepared fraction. On the other hand, it was composed of many mononuclear cells (lymphocytes and monocytes) and a few neutrophils in the Ficoll Paque-prepared fraction, and it was noted that the monocyte percent of this fraction was higher than that of PBL prepared from the IFN-injected mice (36% vs. 20%). Nevertheless, *in vitro* Meth A growth was not inhibited by the PBL of mice co-administered with IFN and G-CSF prepared by using either M-SMF or Ficoll Paque, while it was inhibited by the PBL of mice given IFN.

DISCUSSION

It has been reported that G-CSF can activate not only the growth of hematopoietic progenitor cells *in vitro* and *in vivo*,[6, 8] but also the function of mature neutrophils.[9, 10] Therefore, G-CSF is very useful as an anti-neutropenic agent in cancer therapy in animals and clinical phase studies.[7, 11, 12] However, the effect of G-CSF on the efficacy of cancer therapy, such as tumor regression and prolongation of survival time, remains to be established. In this study, the effect of G-CSF on the IFN therapy of mice i.d. inoculated with Meth A fibrosarcoma was examined to clarify whether or not G-CSF enhances the IFN efficacy. Contrary to our expectation, both the prolongation of survival time and the long-term survival incidence achieved by IFN therapy were reduced by the co-administration of G-CSF (Table I), under conditions where G-CSF protected against neutropenia and leukopenia induced by IFN (Fig. 1).

We have previously reported that IFN generated antiproliferative monocytes in blood of Meth A-bearing and normal mice, suggesting that the production of these cells contributed to the tumor regression.[1] In the present study, the monocyte-enriched fraction derived from the PBL of mice administered with IFN and G-CSF did not inhibit Meth A growth *in vitro*, while that of mice administered IFN alone did (Table II). These results indicated that G-CSF co-administration interfered with the generation of antitumor monocytes by IFN. Alternatively, the antitumor activity of monocytes might be inhibited by G-CSF-induced neutrophils present in the PBL preparation. This inhibitory effect of G-CSF administration on IFN-induced monocytes of PBL may contribute to the reduction of the tumor eradication induced by IFN.

Regarding the processes involved in the inhibitory effect of G-CSF administration on the *in vivo* production and *in vitro* exertion of antitumor activity, we supposed that this inhibitory effect was mediated by neutrophils induced by G-CSF in the host. Although there is no direct evidence for this hypothesis, several reports have described the inhibitory effect of polymorphonuclear neutrophils on antitumor reactions and/or their induction.[13-16] Hence, the similar inhibitory effects produced by the G-CSF-inudced neutrophils possibly affected the antitumor activity of IFN, and conversely, IFN-induced neutropenia was possibly not a side effect but a necessary step for induction of the eradication of Meth A fibrosarcoma by IFN therapy, although further experimentation is required in this regard. It is necessary to determine whether the observed nullifying effect of G-CSF on IFN therapy is also the case with tumors other than Meth A fibrosarcoma.

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