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Predominant Suppression of Neutrophil Colony Growth by Recombinant Human Tumor Necrosis Factor (42601)

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Abstract. To investigate the suppressive effect of recombinant human tumor necrosis factor (rH-TNF) on colony growth of human granulocyte-macrophage progenitor cells (CFU-GM), cytochemical examinations of CFU-GM colonies were performed by a triple staining method. Each colony was classified into five subtypes, and the effects of rH-TNF on each subtype were analyzed. Neutrophil colony growth was inhibited by rH-TNF in a dose-dependent manner, and it was almost completely suppressed at 100 U/ml. In contrast, no significant suppressive effect of rH-TNF was found on the growth of monocyte-macrophage and eosinophil colonies at 100 U/ml or less. When recombinant human granulocyte colony-stimulating factor which almost exclusively stimulates neutrophil colony formation was used as a source of colony-stimulating activity, the total colony growth was almost completely suppressed by 100 U/ml of rH-TNF. These results indicate predominant inhibition of neutrophil colony growth by rH-TNF.

Carswell *et al.* (1) reported the existence of a factor in the serum of bacillus Calmette-Guérin-primed, endotoxin-treated animals that was able to elicit hemorrhagic necrosis of tumors in recipient animals. This factor, referred to as tumor necrosis factor (TNF), has been known as a monocyte product that kills or inhibits the growth of certain tumor cells *in vivo* and *in vitro* (2–5). The recent availability of recombinant human TNF (rH-TNF) (6, 7) had allowed us (8) and others (9, 10) to demonstrate that TNF also inhibited the *in vitro* colony growth of normal human hematopoietic progenitor cells.

To examine this suppressive effect of rH-TNF on human granulocyte-macrophage progenitor cells (CFU-GM) in more detail, we classified granulocyte-macrophage colonies (GM colonies) into several subtypes according to the colony-composing cells by the use of cytochemical stainings, and compared the effects of rH-TNF on each subtype of GM colonies. In this paper, we report that rH-TNF predominantly suppresses the growth of neutrophil colonies (N colonies), but does not significantly suppress that of monocyte-macrophage or eosinophil colonies (Mo or Eo colonies) at the concentrations of 100 units (U)/ml or less.

Materials and Methods. Bone marrow was drawn from five hematologically normal donors and one patient with acute myelogenous leukemia in complete remission. All procedures were performed with informed consent. CFU-GM were assayed according to the method described by Minden *et al.* (11) with a slight modification described previously (8). Briefly, 1×10^5 bone marrow mononuclear cells depleted of T lymphocytes by 2-aminoethylthiouronium bromide-treated sheep red blood cells were plated in 0.5 ml of culture medium containing 0.3% agar (Difco Laboratories, Detroit, MI), α-medium (GIBCO Laboratories, Grand Island, NY), 20% fetal calf serum (GIBCO Laboratories), 10% conditioned medium from phytohemagglutinin-stimulated leukocytes (PHA-LCM) as a source of colony-stimulating activity (CSA), and 1% diluted rH-TNF. In the PHA-LCM used for this study, 1120 U/ml of human interferon (IFN)-γ was detected by a radioimmunoassay kit (Centocor, Malvern, PA). In some experiments, 500 U of recombinant human granulocyte colony-stimulating factor (rH-G-CSF, kindly provided by Kirin, Tokyo, Japan) (12) was also used as a source of CSA instead of PHA-LCM. The rH-G-CSF was produced in *Escherichia coli* which expressed the gene encoding complementary human G-CSF DNA from the subclone IA6 of the human bladder carcinoma cell line 5637 which consistently produces G-CSF. It has a specific activity of 1

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$\times 10^8$ U/mg protein when assayed by serial dilution in a CFU-GM assay. All cultures were performed in triplicate. At the 9th to 11th day of culture (37°C, 5% CO_2), each agar disc was transferred onto a slide glass, fixed in buffered formol acetone (pH 6.6), washed in tap water, and then stained for α-naphtyl butyrate esterase (nonspecific esterase for monocyte-macrophages) and naphthol AS-D chloroacetate esterase (specific esterase for neutrophils) (13), followed by staining with Biebrich scarlet for eosinophils (14). According to the stain findings, each colony (>40 cells) was classified, under a microscope, into five subtypes of GM colonies, i.e., N colonies, Mo colonies, Eo colonies, neutrophil-monocyte-macrophage (NMo) colonies, or neutrophil-eosinophil (NEo) colonies.

rH-TNF was kindly provided by Asahi Chemical Industry, Tokyo, Japan. The rH-TNF was produced in *E. coli* which expressed the gene encoding human TNF that had been identified in a human genomic DNA library (15) by using a cloned complementary DNA encoding a portion of rabbit TNF as a probe (7). The TNF consisted of 155 amino acids with a MW of 17,000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. It has over 99% purity and a specific activity of 2.2×10^6 U/mg protein. The unit was defined as the reciprocal of dilution which killed the 50% of TNF-sensitive mouse L-M cells in the absence of an enhancing agent such as actinomycin D. rH-TNF was diluted with a diluent consisting of 5 m*M* phosphate buffer (pH 7.4), 0.15 *M* NaCl, and 0.1% gelatin, and was added to the culture medium shortly before plating. The dilution was freshly prepared before each experiment.

Statistical analysis was done by the Student *t* test.

Results. As shown in Table I, at the concentrations of 1 to 100 U/ml of rH-TNF, a dose-dependent inhibition of N colonies was found. At the concentrations of 1, 10, and 100 U/ml of rH-TNF, the mean number of N colonies of six experiments decreased to 70 ± 19% (mean ± SD, $P < 0.05$), 16 ± 14% ($P < 0.01$), and 1 ± 1% ($P < 0.01$), respectively, as compared with control cultures without rH-TNF

TABLE I. EFFECT OF rH-TNF ON GROWTH OF THREE SUBTYPES OF HUMAN GRANULOCYTE-MACROPHAGE COLONIES

Case	Age	Sex	Status	Colony	No. of colonies in the presence of rH-TNF (U/ml)[a]			
					0	1	10	100
1	27	M	Normal	N[b]	62 ± 12	31 ± 9*	3**	0**
				Mo[c]	15 ± 7	22 ± 8	22 ± 11	24 ± 6
				Eo[d]	62 ± 13	81 ± 18	93 ± 15*	76 ± 22
2	35	F	Normal	N	27 ± 11	12 ± 2	1 ± 1	<1*
				Mo	12 ± 5	12 ± 5	14 ± 4	19 ± 4
				Eo	15 ± 4	12 ± 1	15 ± 5	10 ± 3
3	30	F	Normal	N	51 ± 15	45 ± 29	4 ± 3	0*
				Mo	11 ± 5	9 ± 1	17 ± 8	15 ± 11
				Eo	46 ± 7	32 ± 12	49 ± 21	62 ± 20
4	18	M	Normal	N	22 ± 14	17 ± 4	3 ± 2	0
				Mo	22 ± 7	13 ± 4	10 ± 2	13 ± 4
				Eo	53 ± 23	57 ± 7	52 ± 15	55 ± 6
5	24	F	Normal	N	78 ± 17	56 ± 10	16 ± 1*	1 ± 1*
				Mo	21 ± 9	13 ± 1	16 ± 5	19 ± 3
				Eo	23 ± 15	34 ± 1	47 ± 1	61 ± 19
6	37	M	A M L[e]	N	34 ± 5	29 ± 9	14 ± 1	<1**
				Mo	8 ± 8	9 ± 4	11 ± 5	12 ± 4
				Eo	12 ± 6	17 ± 1	25 ± 9	28 ± 1*

[a] Mean number of colonies (>40 cells) ±SD per 1×10^5 cells of triplicate cultures.
[b] Neutrophil colony.
[c] Monocyte-macrophage colony.
[d] Eosinophil colony.
[e] Acute myelogenous leukemia in complete remission.
* $P < 0.05$, ** $P < 0.01$.

(100%). Similarly, two neutrophil-containing mixed colonies (NMo and NEo colonies) also seemed to be suppressed in a dose-dependent manner, and at 100 U/ml of rH-TNF, they were almost completely inhibited, although in some cases, the colony numbers of control cultures without rH-TNF were too small to evaluate (data not shown). In contrast, no significant suppression was found on growth of Mo or Eo colonies by rH-TNF. At the concentrations of 1, 10, and 100 U/ml of rH-TNF, the mean numbers of Mo colonies of six experiments were 101 ± 50%, 118 ± 53%, and 131 ± 52%, respectively, and those of Eo colonies were 113 ± 33%, 145 ± 52%, and 154 ± 78%, respectively, as compared with control (100%). When rH-G-CSF was used in CFU-GM culture as a source of CSA instead of PHA-LCM, the colony growth of CFU-GM, exclusively neutrophilic, was almost completely suppressed by 100 U/ml of rH-TNF. Results in Fig. 1 illustrate a representative experiment among three tests.

Discussion. We have shown that rH-TNF predominantly suppresses the *in vitro* colony growth of those progenitor cells which were committed to differentiate to neutrophil-containing colonies at the concentrations of 1 to 100 U/ml. On the other hand, the same concentrations of rH-TNF did not significantly inhibit the growth of Mo or Eo colonies. When rH-G-CSF was used as a source of CSA instead of PHA-LCM, the colony growth of CFU-GM was almost completely suppressed by 100 U/ml of rH-TNF. These findings appear to indicate that rH-TNF predominantly suppresses the growth of N colonies, since rH-G-CSF almost exclusively stimulates N colony formation (12). Broxmeyer *et al.* (10) reported that the suppressive effect of rH-TNF on colony growth of CFU-GM on Day 7 was stronger than that on Day 14. In the light of our present study, their findings might be able to be explained by the predominant inhibitory effect of rH-TNF on colony growth of N progenitors, since on Day 7 of CFU-GM culture, only N colonies are usually found and Mo and Eo colonies grow later than N colonies (16). In contrast to our results and those of Broxmeyer *et al.* (10), Degliantoni *et al.* (9) reported that the suppressive effect of rH-TNF on colony growth of CFU-GM on Day 14 was stronger than that on Day 7. The discordant results might be due to the use of different types or different concentrations of CSF (17).

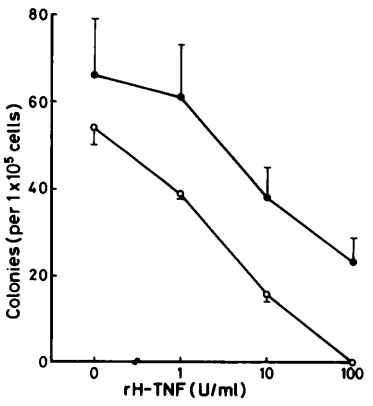

FIG. 1. Comparison of effects of PHA-LCM (●) and rH-G-CSF (○) as a source of colony-stimulating activity (CSA) on colony growth of CFU-GM. Bone marrow cells (1×10^5) from a normal donor were cultured for 9 days in agar gel culture for CFU-GM. Each point with vertical bar represents the mean ± SD of triplicate cultures. When PHA-LCM was used as CSA, subtypes of GM colonies grown in control cultures were 50% (mean) neutrophilic, 18% macrophagic, 19% eosinophilic, 6% neutrophil-macrophagic, and 7% neutrophil-eosinophilic. When rH-G-CSF was used, all GM colonies were neutrophilic.

Since, when PHA-LCM was used as CSA, our assay system for CFU-GM contained 112 U/ml of IFN-γ, one cannot exclude the possibility that IFN-γ in PHA-LCM suppressed the N colony growth of CFU-GM in synergy with rH-TNF (9, 10). However, even when rH-G-CSF was used as CSA, the extent of suppression by rH-TNF on N colony growth was approximately the same as when PHA-LCM was used. Thus, IFN-γ would not have played a major role in the suppression of N colony growth by rH-TNF. Since T lymphocytes were depleted in our experiments, IFN-γ would not have acted indirectly on the progenitor cells through T lymphocytes (18), or a large amount of CSF in our assay system would have overcome the suppressive effect of IFN-γ (19).

The numbers of Mo and Eo colonies appeared to slightly increase in the presence of rH-TNF, although it was not statistically significant. These results suggest a possibility that bipotent GM progenitor cells which normally generate NMo or NEo colonies have been expressed as pure Mo or Eo colonies, respec-

tively, in the presence of rH-TNF. This possibility is supported by our observations that the addition of 100 U/ml of rH-TNF to myelomonocytic leukemia cells caused the change of the nature of colony-composing cells from NMo colonies to pure Mo colonies (unpublished observations), and by others that TNF induced monocytic differentiation of human myeloid cell lines (20).

The present study demonstrates the predominant suppression of N colonies by rH-TNF. Although the mechanism by which TNF inhibits N colony growth remains to be studied, we think our results will contribute to the further study on the differentiation mechanism of granulocyte-macrophage lineage.

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