Induction by recombinant human granulocyte colony-stimulating factor of differentiation of mouse myeloid leukemic M1 cells

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The effect of recombinant human granulocyte colony-stimulating factor (G-CSF) on induction of differentiation of mouse myeloid leukemic M1 cells was examined. Purified G-CSF caused dose-dependent induction of phagocytic activity and lysozyme activity in M1 cells. Its half-maximally effective concentration was 10 ng/ml. On treatment of M1 cells with G-CSF (100 ng/ml) for 4 days, 30–50% of the cells differentiated morphologically into macrophage cells; 30–40% of the cells were blast cells and 20–30% of the cells were forms intermediate between blastic cells and mature macrophages.

Recombinant granulocyte colony-stimulating factor (Myeloid leukemic cell) Differentiation Macrophage Differentiation-inducing factor

1. INTRODUCTION

Mouse myeloid leukemic M1 cells can be induced to differentiate into macrophages by protein inducers (D-factors) and various chemicals [1,2]. On differentiation, M1 cells express morphological, biochemical and functional characteristics of normal macrophages, such as phagocytic and locomotive activities, lysosomal enzyme activities, Fc and C3 receptors and prostaglandin production [1,2]. On differentiation, M1 cells lose their leukemogenicity in syngeneic mice [1], and injection of D-factor preparations into mice inoculated with M1 cells prolongs the survival of the mice [3]. Therefore, inducers of differentiation of myeloid leukemic cells might be effective in the therapy of myeloid leukemia [2,3].

Abbreviations: G-CSF, granulocyte colony-stimulating factor; D-factor, differentiation-inducing factor; cDNA, complementary DNA

We purified the D-factor from conditioned medium of mouse fibroblast L929 cells [4] and mouse Ehrlich ascites tumor cells [5]. Even at higher concentrations, the D-factor did not induce colony formation of macrophages or granulocytes from normal bone marrow cells. Recently, we determined the sequence of the NH_2-terminal amino acids of the factor (unpublished), finding that they differed from those of the CSFs (growth factors for normal precursors of macrophages and/or granulocytes) so far reported.

On the other hand, Nicola et al. [6] reported that the differentiation factor for mouse myelomonocytic leukemic WEHI-3B cells is G-CSF, and Souza et al. [7] showed that recombinant human G-CSF derived from bladder carcinoma 5637 cells induced differentiation of WEHI-3B cells and human leukemic cells. We also cloned a cDNA copy of the gene encoding human G-CSF from a cDNA library constructed from mRNA in macrophages [8]. In the present work, we exam-

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ined the effect of recombinant human G-CSF on induction of the differentiation of M1 cells.

2. MATERIALS AND METHODS

2.1. *Cells and cell culture*

Myeloid leukemic M1 cells originated from a spontaneous myeloid leukemia in an SL mouse [1]. Subclones of M1 cells, clone T22 and newly isolated T22-3 cells were used in the present study. The cells were cultured in Eagle's minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan), supplemented with double the usual concentrations of amino acids and vitamins and 10% (v/v) heat-inactivated (56°C for 30 min) calf serum at 37°C under 5% CO_2 in air.

2.2. *G-CSF*

A cDNA clone of the gene encoding human G-CSF was isolated from a cDNA library constructed from mRNA in human macrophages as described [8]. The amino acid sequence deduced from the cloned G-CSF cDNA was identical with that reported by Souza et al. [7]. The G-CSF cDNA was expressed in *E. coli.* K12 MM294 by construction of an expression plasmid for G-CSF using pGEL-1 containing the *trp* promoter, and the mature G-CSF was purified to homogeneity by the method of Marston et al. [9]. The purified G-CSF (1 mg protein) induced 10^8 granulocyte colonies from human bone marrow cells in agar culture.

2.3. *D-factor*

D-factor was purified to homogeneity from conditioned medium of Ehrlich ascites tumor cells as described [5]. Its specific activity was 6.4×10^7 U/mg protein. 50 U of D-factor were defined as the activity inducing 50% phagocytic cells in 1 ml of M1 cell culture.

2.4. *Cell morphology*

Morphological analysis of M1 cells was performed by the method of Hayashi et al. [10,11]. Cell preparations were made using a cytocentrifuge and the cells were stained with May-Grünwald-Giemsa solution. Microscopic fields were projected onto paper screens at 2000-fold magnification with a Nikon Micropan microprojector, and the perimeters of the cytoplasm and nucleus of individual cells were traced. Each cell image was digitalized with a data tablet digitizer and processed on an on-line minicomputer YHP-2108A. The cell area $S(C)$, nuclear area $S(N)$, and nucleus-cell ratio $NCR = 100 \times S(N)/S(C)$ were calculated.

2.5., *Phagocytosis*

Suspensions of cells (1.2×10^6) in 1 ml of serum-free Eagle's medium containing 0.2% of a suspension of polystyrene latex particles (average diameter, 1.099 μm; Dow Chemical Co., Indianapolis, IN) were incubated for 4 h at 37°C. Then the cells were thoroughly washed 3 times with phosphate-buffered saline, and the percentage of phagocytic cells in more than 200 cells was determined. Cells containing more than 10 latex particles were scored as phagocytic cells.

2.6. *Lysozyme*

Lysozyme activity in the cells was determined by the lysoplate method of Osserman and Lawlor [12] as modified by Kasukabe et al. [13]. The activity, in microgram equivalents of hen egg white lysozyme, was calculated from a standard curve prepared using purified hen egg white lysozyme. The results are expressed as amounts of lysozyme in microgram equivalents per mg of cell protein.

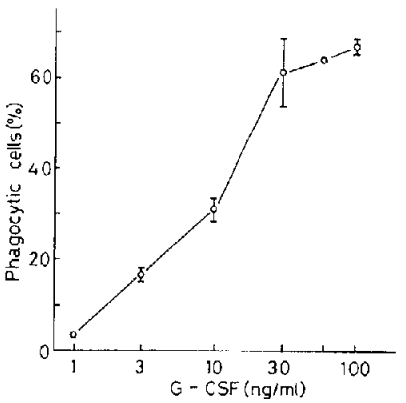

Fig.1. Induction of phagocytic activity in M1 cells by G-CSF. M1 cells were incubated for 2 days with various concentrations of G-CSF and then their phagocytic activity for polystyrene latex particles was assayed. Points and bars are means ± SE for duplicate determinations.

3. RESULTS

3.1. *Induction of the differentiation of M1 cells by G-CSF*

Recombinant human G-CSF induced phagocytic activity, a typical marker of cell differentiation in M1 cells. G-CSF caused dose-dependent induction and its half-maximal concentration was 10 ng/ml (fig.1). The proportion of phagocytic cells increased with increase in the incubation time for 4 days (fig.2). G-CSF also induced lysozyme activity in M1 cells; lysozyme activity in the cells treated with 100 ng G-CSF per ml for 4 days was 4.1 U/mg protein, whereas that in untreated M1 cells was 0.4 U/mg protein.

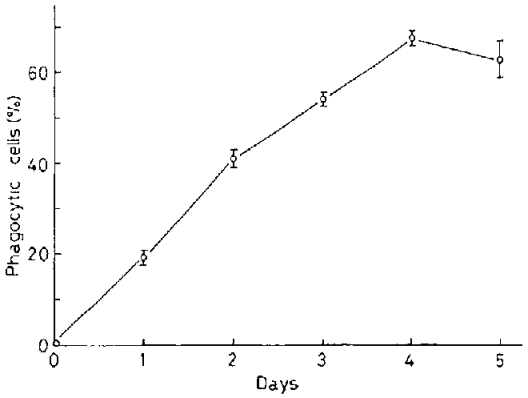

Fig.2. Induction of phagocytic activity in M1 cells after different times of incubation with G-CSF (60 ng/ml). Points and bars are means ± SE for duplicate determinations.

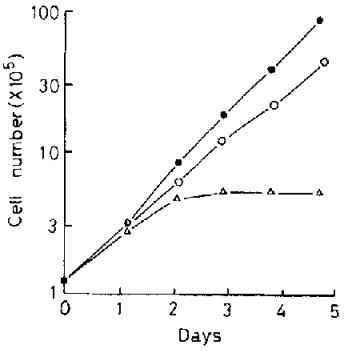

Fig.3. Growth of M1 (T22-3 clone) cells. Cells were cultured in the presence of 60 ng/ml of G-CSF (○) or 500 U/ml of D-factor (△) per ml or in the absence of factors (●). On day 3, cultures were diluted with fresh medium to avoid nutrient depletion. Cell numbers were counted with a Coulter counter. Values indicate cumulative cell numbers.

Culture of M1 cells in the presence of G-CSF resulted in slight reduction in proliferation of the cells (fig.3), but increase in the cell number did not stop even after their treatment with G-CSF for 7 days. Cell morphology was examined by staining the cells with May-Grünwald-Giemsa solution. As shown in fig.4a, untreated M1 cells had a large round or slightly indented nucleus and little

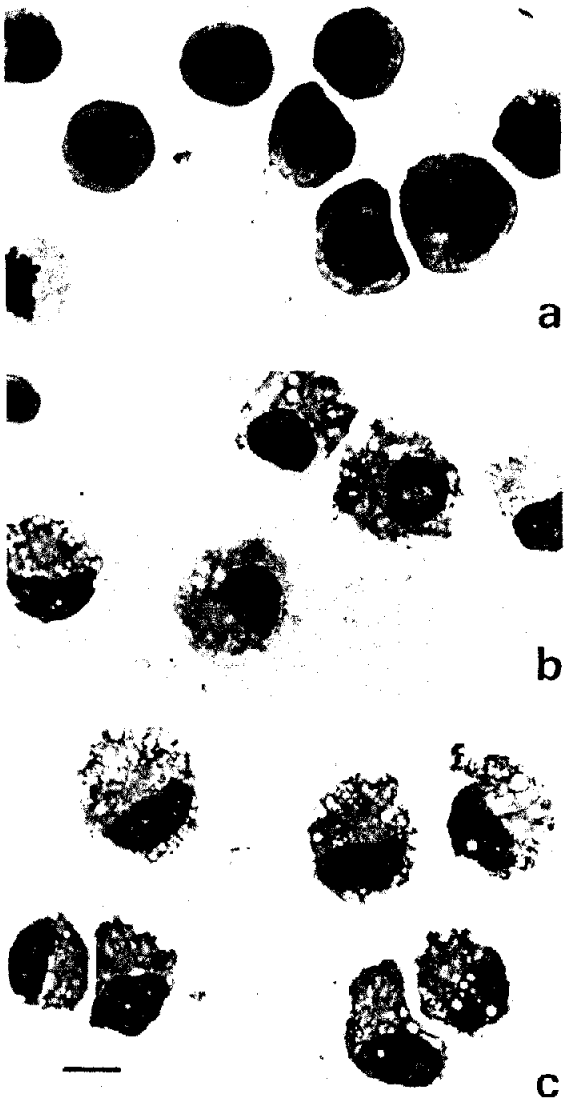

Fig.4. Morphology of M1 cells stained with May-Grünwald-Giemsa stain. The bar represents 10 μm. (a) Untreated clone T22-3 cells; (b) cells treated with G-CSF (100 ng/ml) for 4 days; (c) cells treated with D-factor (500 U/ml) for 4 days.

cytoplasm. After treatment with G-CSF for 4 days, 60–70% of the cells changed morphologically; 30–50% of the cells were similar to macrophages, as shown in fig.4b, while 20–30% were forms intermediate between blastic cells and mature macrophages. The nucleus-cell ratio of individual cells was determined and the frequency distributions of the cells are shown in fig.5. On differentiation, M1 cells showed decrease in the nucleus-cell ratio.

However, even a higher concentration of G-CSF (200 ng/ml) did not induce phagocytic activity in clone R1 cells [14], a subclone of M1 cells that was resistant to induction of differentiation by D-factor (10000 U/ml).

3.2. *Comparison of G-CSF and D-factor*

Under the same conditions we examined the effects of D-factor and G-CSF on the induction of differentiation and growth of M1 cells. The maximum percentage of phagocytic cells induced by G-CSF was 60–70% (figs 1 and 2). On the other hand, D-factor purified from conditioned medium of L929 cells or Ehrlich ascites tumor cells induced phagocytic activity in more than 90% of the M1 cells [4,5]. On treatment of M1 cells with D-factor purified to homogeneity from conditioned medium of Ehrlich ascites tumor cells, more than 99% of the M1 cells differentiated into macrophages (figs 4c and 5) and growth of the cells ceased after 2 days (fig.3). The nucleus-cell ratios of the cells treated with D-factor for 4 days were less than 40%, while those of cells treated with G-CSF were widely distributed between 20 and 70% (fig.5).

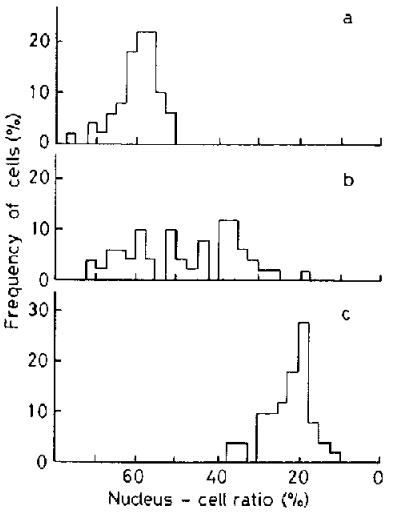

Fig.5. Morphological analysis of M1 cells. (a) Untreated clone T22-3 cells; (b) cells treated with G-CSF (100 ng/ml) for 4 days; (c) cells treated with D-factor (500 U/ml) for 4 days. The nucleus-cell ratio of cells was determined as described in section 2 on 50 randomly selected cells.

4. DISCUSSION

In this work, we showed that recombinant human G-CSF induced differentiation of mouse myeloid leukemic M1 cells into cells that were functionally, biochemically and morphologically similar to macrophages. The half-maximal concentration of G-CSF for inducing differentiation of M1 cells was 10 ng/ml, or 5.3×10^{-10} M, since the molecular mass of G-CSF deduced from the amino acid sequence is 18700 Da. On the other hand, the half-maximally effective concentration of D-factor on M1 cells was 2×10^{-11} M [4,5]. Souza et al. [7] reported that recombinant human G-CSF showed activity on both human bone marrow cells and a mouse myelomonocytic leukemia cell line WEHI-3B with a half-maximally effective concentration of 2.7×10^{-11} M. Therefore, the action of G-CSF is not species specific. Although G-CSF can support growth of mixed colony progenitors (colony-forming unit-granulocyte, erythroid, macrophage and megakaryocyte), it mainly stimulates growth and function of granulocytes in normal hematopoietic cells [7]. Therefore, the low sensitivity of M1 cells to G-CSF may be associated with the inherent character of M1 cells to differentiate predominantly into macrophages.

Even under the optimum conditions of differentiation of M1 cells induced by G-CSF, 30–40% blastic cells remained. The nucleus-cell ratios of these cells were more than 50%. Hayashi et al. [10,11] examined the relation between the nucleus-cell ratio of M1 cells and the ability of the cells to proliferate. They showed that differentiated M1 cells in which the nucleus-cell ratio decreased to below 40% lost the ability to enter the S phase of the cell cycle. The nucleus-cell ratios of more than 99% of the cells treated with D-factor were less than 40%, whereas those of cells treated with G-CSF were widely distributed between 20 and 70% (fig.5). These results are consistent with results on

the effects of G-CSF and D-factor on growth of M1 cells (fig.3). Growth of M1 cells was suppressed completely by D-factor and partially by G-CSF.

We previously showed that there are two types of D-factor for M1 cells, which differ from each other in antigenicity and molecular size [5,13]. The D-factor with a molecular mass of 40000–70000 Da is produced by mouse L929 cells, Ehrlich ascites tumor cells and activated lymphocytes [15,16], and a purified preparation of this D-factor did not show CSF activity [5,16]. The other D-factor with a molecular mass of 20000–25000 Da is produced by macrophages and has not yet been purified. G-CSF is also produced by macrophages [17] and the molecular mass of its glycosylated form is 25000 Da [6]. Therefore, the D-factor for M1 cells produced by macrophages might be identical to G-CSF. Elucidation of this problem should be facilitated by the availability of antibody to G-CSF.

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