Enhancement of the Antibody-dependent Tumoricidal Activity of Human Monocytes by Human Monocytic Colony-stimulating Factor

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Human monocytic colony-stimulating factor (hM-CSF) stimulates mature cells as well as progenitor cells of human monocyte lineage. In the present study, we investigated the effect of completely purified native hM-CSF on the tumoricidal activity of human monocytes against Raji (Burkitt lymphoma-derived cell line) using a chromium release assay. Preincubation of human peripheral blood monocytes with hM-CSF for 48 h markedly enhanced their antibody-dependent cellular cytotoxicity (ADCC). This enhancing effect was dependent on the concentration of hM-CSF, and maximal enhancement was achieved at the concentration of 66 ng/ml. The number of receptor sites on monocytes against the Fc portion of immunoglobulin G increased 2- to 4-fold during 48 h incubation with hM-CSF. These results suggest that hM-CSF stimulates monocyte-ADCC by increasing the number of Fc receptor sites on human monocytes.

Key words: Human monocytic colony-stimulating factor — Monocyte — Antibody-dependent cellular cytotoxicity

Human monocytic colony-stimulating factor (hM-CSF) is one of the hematopoietic growth factors that regulate the proliferation and differentiation of blood cells.^{1,2} Two types of hM-CSFs were purified from human urine, and their cDNAs have been cloned.^{3,4} One has an apparent molecular weight of 45kD (smaller type) and the other, 85kD (larger type).⁵ The larger type of hM-CSF has an additional unique amino acid sequence at the carboxyl terminal that is not found in the smaller type of hM-CSF.^{3,4}

Human M-CSF has a macrophagic colony-stimulating activity, and it promotes the release of a number of soluble factors from mature monocytes such as granulocyte CSF (G-CSF),⁶ granulocyte-macrophage CSF (GM-CSF),⁶ tumor necrosis factor (TNF),⁷ and interferon (IFN).⁷ In addition, it stimulates the effector functions of mature monocyte/macrophages including the antibody-independent and antibody-dependent tumoricidal activity,^{8–10} and anti-viral activity of murine macrophages.¹¹ It was also reported that murine M-CSF induced macrophages to inhibit the proliferation of tumor cells,¹² to enhance the tumoricidal activity induced by lymphokine,¹³ and to enhance the killing of *Candida albicans*.¹⁴ In this paper, we describe the enhancing effect of the native hM-CSF on the antibody-dependent cellular cytotoxicity (ADCC) mediated by human monocytes against human leukemic cell line, Raji.

MATERIALS AND METHODS

Reagents The larger type of hM-CSF was purified from human urine.¹⁵ The final material from the purification procedure was homogeneous on SDS-polyacrylamide gels, and exhibited a single amino-terminal amino acid. It had a specific activity of 1.5×10^8 U/mg protein when assayed in the standard mouse assay system.¹⁶ The endotoxin content of the purified hM-CSF fraction was less than 0.01 ng in 1 ml which contained 66 ng of hM-CSF as measured by the *Limulus* assay (*Limulus* HS-Single Test; Wako Pure Chemicals, Osaka). Antiserum against Raji cells (Burkitt lymphoma-derived cell line) was prepared by immunizing rabbits with Raji cells. The immunoglobulin G (IgG) fraction was prepared from the antiserum, and its purity was more than 90% on SDS-polyacrylamide gel. Both the antiserum and IgG fraction contained less than 0.1 ng of endotoxin at the concentration we used. All cell cultures were performed in RPMI 1640 medium (Flow Laboratories, McLean, VA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Filtron Ltd., Victoria, Australia). This complete medium contained less than 0.1 ng of endotoxin in 1 ml. Lipopolysaccharide (LPS) and polymyxin B were purchased from Sigma (St. Louis, MO).

Isolation and preincubation of human monocytes Peripheral blood cells were obtained from healthy donors who had given informed consent, and mononuclear cells were separated by density gradient centrifugation on Ficoll/

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Isopaque solution (Lymphoprep; Nycomed, Oslo, Norway). The mononuclear cells were then washed at least three times with Hanks' balanced salts solution, seeded on 10 cm plastic dishes and incubated at 37°C for 2 h. After removal of the non-adherent cells by vigorous washing with RPMI 1640 medium, the adherent cells were detached by gentle scraping. The purity of the monocyte preparations was usually more than 90% and 95% as determined by morphologic (Wright-Giemsa staining) and enzymatic (nonspecific esterase staining) analyses, respectively, of the cytocentrifuged specimen. Monocytes were resuspended in the complete medium at a concentration of 3.0×10^6 cells/ml and incubated in the presence or absence of various concentrations of hM-CSF before and after heat treatment (100°C, 3 min) for 1–3 days in polypropylene tubes (Falcon, No. 2059, Becton Dickinson Labware, Lincoln Park, NJ) before measuring the cytotoxicity or Fc receptor number.¹⁷⁾

Cytotoxicity assay Monocyte cytotoxicity was measured using a chromium (Cr) release assay in which ⁵¹Cr-labeled Raji cells were used as the target cells. Raji cells in log phase growth were labeled with ⁵¹Cr (NEN, Boston, MA) for 1 h at 37°C at a concentration of 100 μ Ci/ 10^6 cells. The effector monocytes, which had been washed with RPMI 1640 medium and resuspended in the complete medium, were mixed with 3×10^3 ⁵¹Cr-labeled target Raji cells to give an effector:target (E:T) ratio of 10:1 to 100:1 in a final volume of 0.2 ml/well, in flat-bottomed, 96-well plates (Nunc, Roskilde, Denmark). Heat-inactivated anti-Raji antiserum at 1:1,000 dilution or anti-Raji IgG at a concentration of 0.2 to 5.0 μ g/ml was added to the assay mixture for measuring the ADCC activity. The plates were incubated for 12 h at 37°C, then 0.1 ml of supernatant aspirated from each well was counted in a gamma counter, and the percent (%) cytotoxicity was calculated using the following formula:

$$\begin{aligned} & \% \text{ cytotoxicity} \\ &= \frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{maximal release (cpm)} - \text{spontaneous release (cpm)}} \times 100. \end{aligned}$$

Spontaneous release was determined by incubating target cells in the complete medium. The anti-Raji serum and anti-Raji IgG did not show any tumor cell killing activity towards Raji cells, and the percentage of spontaneous ⁵¹Cr release was always 15% or less. Maximal release was determined using the supernatant after lysing the Raji cells with 1% SDS. Data are expressed as mean value $\pm$ standard deviation (SD) of triplicate assays.

Fc receptor assay Monoclonal rabbit IgG-coated sheep erythrocytes (EA) were purchased from The Binding Site, Ltd. (Birmingham, UK). Monocytes incubated in the presence or absence of hM-CSF were placed in 96-

well plates at a concentration of 1.5×10^5 cells/0.1 ml per well. After incubation of the plates for 2 h at 37°C, 0.1 ml of 2.5% EA suspension was added to each well, and this mixture was further incubated at 4°C for 1 h with gentle agitation. The cells were then washed five times with RPMI 1640 medium to remove unattached EA. To visualize rosettes, cells were fixed in methanol and stained with Wright-Giemsa.¹⁸⁾ Student's *t* test was used to compare the data in the presence of hM-CSF with the data in its absence.

RESULTS

Effect of hM-CSF on monocyte-ADCC Antibody-independent and antibody-dependent cytotoxicities of human peripheral blood monocytes were tested after preincubating them with or without hM-CSF for 1–3 days using ⁵¹Cr-labeled Raji cells as the target cells. Figure 1 shows a representative example of four experiments. Human monocytes did not show any killing activity against Raji cells in the absence of anti-Raji IgG (antibody-independent cellular cytotoxicity), which was usually 5% or less, and was not stimulated by preincubation with hM-CSF. On the other hand, human monocytes showed a moderate ADCC (spontaneous ADCC), and they showed a marked ADCC after preincubation with hM-CSF. Human monocytes showed 31, 27 and 36% cytotoxicity after 1, 2 and 3 days of preincubation in the

Fig. 1. Antibody-independent and antibody-dependent cytotoxicities of human peripheral blood monocytes against Raji cells. Monocytes were preincubated in the absence (□) or presence (■) of 66 ng/ml hM-CSF and assayed for cytotoxicity in the absence or presence of 5 μ g/ml anti-Raji IgG at an E:T ratio of 100:1. Bars indicate SD.

Fig. 2. Effect of hM-CSF on antibody-independent and antibody-dependent cytotoxicities of monocytes against Raji cells. Monocytes were incubated with 0–66 ng/ml (Experiment 1) or 0–333 ng/ml (Experiment 2) hM-CSF for 2 days and assayed for cytotoxicity in the presence of 1,000-fold-diluted normal serum (○) or 1,000-fold-diluted anti-Raji serum (●), at an E:T ratio of 100:1. Bars indicate SD.

absence of hM-CSF, respectively, and 37, 57 and 58% cytotoxicity after the same periods of preincubation in the presence of 66 ng/ml hM-CSF, respectively. A 2-day preincubation was required and sufficient for the maximal stimulation of monocyte-ADCC.

Figure 2 shows the dose-response relation between the concentration of hM-CSF in the preincubation mixture and the percent cytotoxicity in the ADCC assay, based on two representative examples of nine experiments. Enhancement of monocyte-ADCC induced by hM-CSF was dose-dependent, and the maximal ADCC activity was observed when monocytes were preincubated with 66 ng/ml hM-CSF.

We next examined the effect of anti-Raji IgG concentration and E:T ratio on monocyte-ADCC as shown in Table I (representative example of three experiments). Clear enhancement of monocyte-ADCC by hM-CSF was observed at E:T ratios of 100:1 and 50:1, and at concentrations of anti-Raji IgG of more than 1 μ g/ml.

Then, the effects of LPS and polymyxin B on monocyte-ADCC were examined (Table II; a representative example of two experiments). Lipopolysaccharide up to 1 μ g/ml did not stimulate monocyte-ADCC and did not enhance monocyte-ADCC induced by hM-CSF. The addition of polymyxin B to the preincubation mixture did not reduce the ADCC of monocytes preincubated with medium alone or with 66 ng/ml hM-CSF. Heat-in-

Table I. Effect of Anti-Raji IgG Concentration and E:T Ratio on Monocyte-ADCC

E/T ratio	Preincubation with	Anti-Raji IgG (μ g/ml)			
		0	0.2	1.0	5.0
100	medium	4 $\pm$ 2	4 $\pm$ 1	9 $\pm$ 1	23 $\pm$ 1
	hM-CSF	5 $\pm$ 1	7 $\pm$ 2	49 $\pm$ 3	63 $\pm$ 2
50	medium	1 $\pm$ 1	3 $\pm$ 1	3 $\pm$ 1	10 $\pm$ 2
	hM-CSF	2 $\pm$ 0	1 $\pm$ 1	20 $\pm$ 1	30 $\pm$ 1
10	medium	0 $\pm$ 1	–1 $\pm$ 1	0 $\pm$ 1	1 $\pm$ 0
	hM-CSF	–1 $\pm$ 0	–1 $\pm$ 1	3 $\pm$ 0	4 $\pm$ 1

Human monocytes were preincubated with medium alone or with 66 ng/ml hM-CSF for 2 days and assayed for cytotoxicity in the absence or presence of 0.2, 1.0 and 5.0 μ g/ml of anti-Raji IgG, at E:T ratios of 100:1, 50:1 and 10:1. Data indicate the percent cytotoxicity expressed as the mean $\pm$ SD of triplicate assays.

Table II. Effects of the Addition of LPS and Polymyxin B to the Preincubation Mixture on Monocyte-ADCC

Preincubation with	1.0 μ g/ml polymyxin B	
	–	+
medium	27 $\pm$ 3	27 $\pm$ 3
LPS (0.1 μ g/ml)	21 $\pm$ 3	25 $\pm$ 1
LPS (1.0 μ g/ml)	23 $\pm$ 1	25 $\pm$ 2
hM-CSF (66 ng/ml)	54 $\pm$ 2	48 $\pm$ 1
hM-CSF + LPS	55 $\pm$ 4	56 $\pm$ 5
heated hM-CSF	25 $\pm$ 4	27 $\pm$ 2

Human monocytes were preincubated for 2 days with medium, 0.1 μ g/ml or 1.0 μ g/ml LPS, 66 ng/ml hM-CSF, 66 ng/ml hM-CSF plus 1.0 μ g/ml LPS, or heat-inactivated (100°C, 3 min) hM-CSF, in the absence or presence of 1.0 μ g/ml polymyxin B. ADCC assay was performed in the presence of 5.0 μ g/ml anti-Raji IgG, at an E:T ratio of 100:1. Data indicate the percent ADCC expressed as the mean $\pm$ SD of triplicate assays.

activated hM-CSF did not show any enhancing effect on monocyte-ADCC.

The data from 18 monocyte donors are summarized in Fig. 3. The spontaneous ADCC of monocytes preincubated with medium alone for 2 days varied from 10% to 42%, and the ADCC of monocytes after a 2-day preincubation with 66 ng/ml hM-CSF varied from 21% to 80%. The average value of hM-CSF-dependent ADCC was 53.4 $\pm$ 13.9%, which was significantly higher than that of spontaneous ADCC (26.3 $\pm$ 11.0%) with $P < 0.01$.

Effect of hM-CSF on Fc receptor expression To evaluate the effect of hM-CSF on Fc receptor (FcR) expression on the monocyte surface, monocytes were incubated for 1–2 days with medium alone or 33 ng/ml hM-CSF and

Fig. 3. Antibody-dependent cytotoxicity (ADCC) of monocytes obtained from 18 healthy volunteers. Monocytes were preincubated with or without 66 ng/ml hM-CSF for 2 days and then assayed for the ADCC in the presence of 5 μ g/ml of anti-Raji IgG at an E:T ratio of 100:1.

Fig. 4. Expression of Fc receptor sites on monocytes. Monocytes were incubated in the absence (a) or presence (b) of 33 ng/ml hM-CSF for 1 or 2 days and then mixed with sheep erythrocytes combined with anti-sheep erythrocyte rabbit IgG (EAs).

mixed with sheep erythrocytes bound with monoclonal IgG (EA). Figure 4 shows the result of rosette formation (a representative example of five experiments). Incubation with hM-CSF for 2 days dramatically increased FcR-mediated binding of EA to monocytes. No increased binding was observed after 1 day of incubation. A hundred monocytes were counted to determine the number of EA attached to each monocyte in three separate experiments according to the method reported previously.¹⁸⁾ Although the average number of EA of monocytes incubated with 33 ng/ml hM-CSF for 2 days was variable from 2.7 ± 3.1 to 6.5 ± 3.7 , it was always more than that after the 2-day incubation without hM-CSF (from 1.5 ± 2.0 to 2.0 ± 1.9 EAs/monocyte), with statistical significance ($P < 0.01$). The maximal FcR expression was observed at a concentration of 66 ng/ml hM-CSF (from 3.2 ± 2.7 to 7.3 ± 4.5 EAs/monocyte).

DISCUSSION

In this study, we have demonstrated that the native hM-CSF stimulates the ADCC of human monocytes against Raji cells. The maximal enhancement by hM-CSF was achieved at a concentration which was comparable to the concentration needed to stimulate the monocyte production of G-CSF and GM-CSF.⁶⁾ Although hM-CSF induces monocyte survival,¹⁹⁾ this might not be a major factor in the ADCC-enhancing effect because monocytes were resuspended to give the same viable cell number immediately before the ADCC assay after preincubation as described in "Materials and Methods." The monocyte preparations were not completely pure and we could not completely exclude the possibility that the contaminating cells in our monocyte fractions were involved in the antibody-dependent killing of target cells.

We have also demonstrated that hM-CSF augmented IgG FcR expression on human monocytes during the 2-day incubation. It had been reported that the smaller type of hM-CSF enhanced FcRI expression on human monocytes,¹⁹⁾ and murine M-CSF upregulated the expression of FcRI and II on murine macrophages.¹⁸⁾ It was also reported that at least FcRI and II were important in mediating monocyte-ADCC.²⁰⁾ In the present study, a rabbit polyclonal antibody was used in the ADCC assay, and we did not determine which type of FcRs was upregulated by the larger type of hM-CSF. However, ADCC-related FcR might be involved because the period of preincubation with hM-CSF and the amount of hM-CSF required to obtain maximal enhancement of monocyte-ADCC were identical to those required to obtain maximal expression of FcR sites on the monocyte surface. As shown in Fig. 2, the enhancement in ADCC was observed only when anti-Raji antiserum was added

to the assay mixture, indicating that the binding of anti-Raji antibody increased after treatment of monocytes with hM-CSF.

Earlier studies demonstrated the augmentation of neutrophil ADCC by G-CSF and GM-CSF.²¹⁻²³) Nakoinz and Ralph reported that murine M-CSF and the smaller type of human M-CSF alone had little effect on the ADCC of murine macrophages against murine T-lymphoma cells R1.1, but they strongly stimulated the ADCC when they were combined with interferons and interleukin 2.⁹) Mufson *et al.* reported that the recombinant larger type of hM-CSF was effective at enhancing the ADCC of human monocytes and murine macrophages against human colon carcinoma cells LS-174.¹⁰) Although our data presented here confirmed their observation that hM-CSF was an effective stimulator of monocyte-ADCC, there are some differences in pre-incubation period and concentration of hM-CSF between our experiment and theirs. The effects of assay procedure and source of hM-CSFs might account for these differences. The mechanisms of enhanced ADCC activity by various CSFs remain to be established in detail.

In this study, we did not observe the enhancing effect of hM-CSF on the antibody-independent tumor cell killing of Raji cells. However, other investigators showed that hM-CSF was an effective stimulator of the antibody-independent tumoricidal activity of monocytes.⁸) Recently, we have also observed that the native hM-CSF boosted monocyte tumoricidal activity against several human leukemic cells.²⁴) Thus, hM-CSF seems to be a positive regulator of both antibody-independent and antibody-dependent tumoricidal activities of human monocytes. An *in vivo* study in which tumor-bearing mice would be injected with hM-CSF is under consideration to confirm these *in vitro* observations.

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