ABSTRACT

Human monocytic colony-stimulating factor (hM-CSF) enhances several effector functions of human peripheral blood monocytes. In this study, we investigated the effect of the M, 85,000 form of hM-CSF on the tumoricidal activity of human monocytes against several leukemic cell lines using a 12-h chromium release assay. Human peripheral blood monocytes preincubated with hM-CSF for 48 h showed more effective killing activity towards K562, U937, Daudi, and HL60 cells as compared with the cells preincubated with medium alone. Maximal enhancement of the tumoricidal activity was achieved by hM-CSF at concentrations of 50–100 ng/ml. A trace amount of lipopolysaccharide contained in the hM-CSF did not seem to contribute to the enhancing effect, as the addition of a lipopolysaccharide-neutralizing agent, polymyxin B, to the preincubation mixture did not reduce this effect. Anti-tumor necrosis factor antiserum partially blocked the tumoricidal activity mediated by hM-CSF, indicating that tumor necrosis factor may participate in the hM-CSF-mediated increase of monocyte tumor-killing activity. These results suggest that in addition to other monocyte-activating factors, hM-CSF augments monocyte tumoricidal activity against a wide spectrum of tumor targets.

INTRODUCTION

Human monocyte/macrophages are vital effector cells in host defense against malignant neoplasia. Although they exhibit spontaneous tumoricidal activity, marked tumor lysis can be achieved if they are activated with several agents. IFN-γ is a well-known monocyte-activating factor (1, 2), but there is a considerable amount of evidence indicating that a number of cytokines such as GM-CSF and interleukins 2, 3, and 4 have similar activities in human or murine systems (3–7).

hM-CSF is a homodimeric glycoprotein, and two forms of hM-CSF have been well characterized: One has an apparent molecular weight of 45,000 and the other, 85,000 (8–10). hM-CSF has macrophage colony-stimulating activity and acts as a growth factor for monocyte progenitors in vitro (8, 9). It also enhances several effector functions of mature monocytes and promotes the monocyte production of a number of soluble factors including G-CSF, GM-CSF, TNF, and IFN (11–13). Increased production of TNF and IFN might enhance monocyte tumor-killing activity.

We have examined whether the M, 85,000 form of hM-CSF can enhance direct cytotoxicity by human monocytes. Recent studies from our group1 and others show that hM-CSF stimulates antibody-dependent tumoricidal activity of mature monocytes (13, 14). In this communication, we report the enhancing effect of hM-CSF on monocyte antibody-independent tumoricidal activity against several human leukemic cell lines: K562, U937, Daudi, and HL60 cells.

MATERIALS AND METHODS

Reagents. The M, 85,000 form of hM-CSF was purified from human urine as previously described (15). The final material from the purification procedure was homogeneous as judged by SDS-polyacrylamide gel electrophoresis and amino-terminal amino acid determination. It had a specific activity of 1.8 x 10^4 units/mg protein when assayed in the standard mouse assay system (16). All cell cultures were performed in RPMI 1640 medium (Flow Laboratories, McLean, VA) supplemented with 10% heat-inactivated FCS (Filtron Ltd., Victoria, Australia). The endotoxin content of the purified hM-CSF fraction used in this study was less than 0.01 ng in 1 ml which contained 50 ng hM-CSF, and the culture medium containing 10% FCS had less than 0.1 ng endotoxin in 1 ml as measured by the Limulus assay (Limulus HS-Single Test; Wako Pure Chemicals, Osaka, Japan). Polymyxin B was purchased from Sigma (St. Louis, MO). Anti-recombinant human TNF rabbit antiserum was purchased from Genzyme (Boston, MA) and had an approximately 5 x 10^4 neutralizing units per milliliter.

Cell Lines. The four cell lines used in this study were K562, an erythroleukemia cell line; U937, a monocyte-like cell line; Daudi, a B-lymphoblast cell line; and HL60, a promyelocytic leukemia cell line. Each was maintained in a suspension culture containing the complete medium (RPMI 1640 medium supplemented with 10% FCS), and the medium was changed two or three times a week.

Isolation of Human Monocytes. Peripheral blood was obtained from healthy donors who had given their informed consent. Mononuclear cells were separated by density gradient centrifugation on Ficoll/Isopaque solution (Lymphoprep; Nycomed, Oslo, Norway). They were washed at least three times with Hanks’ balanced salt solution, resuspended in RPMI 1640 medium containing 2.5% FCS, and seeded on 10-cm plastic dishes. After incubating at 37°C for 2 h, the nonadherent cells were removed by vigorous washing with RPMI 1640 medium, and the adherent cells were detached by gentle scraping. The purity of the monocyte preparations was usually more than 90% as determined by morphologic (Wright-Giemsa staining) and enzymatic (nonspecific esterase staining) analyses of the cytocentrifuged specimen. Monocytes were resuspended in the complete medium at a concentration of 1.0–3.0 x 10^6 cells/ml and incubated in the absence or presence of various concentrations of hM-CSF for 0–3 days in polypropylene tubes (Falcon, No. 2059, Becton Dickinson Labware, Lincoln Park, NJ). 51Cr (NEN, Boston, MA) was added to the cells at a concentration of 100 μCi/10^6 cells for 1 h at 37°C. Effector monocytes were washed with RPMI 1640 medium, resuspended in the complete medium which did not contain hM-CSF as described above, and mixed with 3
× 10³¹⁷Cr-labeled target cells to yield E:T ratios of 10–100:1, in a total volume of 0.2 ml/well in a flat-bottomed, 96-well plate (Nunclon, Roskilde, Denmark). The effector/target mixture was further incubated at 37°C for 12 h. Cell-free supernatant (0.1 ml) was collected from each well by centrifugation at 1,000 × g for 5 min and then counted for radioactivity in a gamma counter. The percentage cytotoxicity was calculated from the following formula:

\[
\% \text{ cytotoxicity} = \frac{\text{Experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{Maximal release (cpm)} - \text{spontaneous release (cpm)}} \times 100
\]

Spontaneous release was determined by incubating the target cells in the complete medium, and the percentage of spontaneous release was always within 20% for all types of target cells. Maximal release was determined using the supernatant after lysing the target cells with 1% SDS. Data were expressed as a mean value ± standard deviation for duplicate assays. Comparison of the cytotoxicity was done using Student's t test.

**RESULTS**

To evaluate the effect of hM-CSF on monocyte tumoricidal activity, human peripheral blood monocytes were preincubated with 50 ng/ml hM-CSF for 0–3 days and measured for their lytic activity towards several human leukemic cell lines: K562, U937, Daudi, and HL60 cells, using a chromium release assay. Fig. 1 illustrates the changes in the tumoricidal activity of monocytes when they were preincubated with 50 ng/ml hM-CSF for various incubation periods. Preincubation of monocytes with hM-CSF for 1 day was not sufficient to obtain the maximal enhancement of tumoricidal activity. Monocytes preincubated with hM-CSF for 2 days showed 2- to 6-fold higher tumoricidal activity against all target cells as compared with the activity of control cells incubated with medium alone. The data on the cells preincubated for 3 days were similar to those of the 2-day-preincubated cells, indicating that 2-day preincubation is sufficient for maximal enhancement.

We examined the dose-response between the concentration of hM-CSF in the preincubation mixture and the percentage of tumoricidal activity after a 2-day preincubation using K562 and U937. Fig. 2 shows that hM-CSF enhanced monocyte tumoricidal activity in a dose-dependent manner. Maximal stimulatory effect was observed when the monocytes were preincubated with 50–100 ng/ml hM-CSF. A similar dose-response curve and a similar optimal dose of hM-CSF were observed when Daudi and HL60 cells were used as targets (data not shown).

Maximal enhancement of the tumoricidal activity was observed when the number of monocytes added in the chromium release assay was 50-fold or more than the number of target cells (Fig. 3). Although the level of the enhancement of the tumoricidal activity of monocytes by hM-CSF varied from donor to donor, statistically significant enhancement was observed at an E:T ratio of 50:1 or more, but not at an E:T ratio of 10:1. HL60 cells were more resistant against monocyte cytotoxicity, and the enhancing effect of cytotoxicity by hM-CSF was observed only at an E:T ratio of 100:1.

Although the content of LPS contaminating all reagents used in this study was 0.1 ng/ml or less, the tumoricidal activity of monocytes preincubated with hM-CSF was compared with that of cells preincubated with both hM-CSF and polymyxin B to reduce the possibility that LPS may contribute to the enhancement of monocyte tumoricidal activity. As shown in Fig. 4, the addition of polymyxin B did not alter the baseline cytotoxicity, and it did not reduce the enhancing effect of hM-CSF.

The tumoricidal activities of monocytes obtained from all donors are summarized in Fig. 5. Although the level of the enhancement was variable from donor to donor, hM-CSF augmented monocyte cytotoxicity against all types of target cells.

**Fig. 1.** Tumoricidal activity of human peripheral blood monocytes after preincubation with hM-CSF for various periods. Monocytes were preincubated with 50 ng/ml hM-CSF for 0–3 days and assayed for their cytotoxicity against K562, U937, Daudi, and HL60 cells. The E:T ratio was 50:1 in the cases of K562, U937, and Daudi cells and 100:1 in the case of HL60 cells. Data are shown as the mean ± SD of two separate experiments.

**Fig. 2.** Tumoricidal activity of human peripheral blood monocytes after preincubation with various concentrations of hM-CSF. Monocytes were preincubated with hM-CSF for 2 days and assayed for their cytotoxicity against K562 cells (B) and U937 cells (A) at an E:T ratio of 50:1. Data are shown as the mean ± SD of two separate experiments.
Hm-CSF and Monocyte Cytotoxicity

in almost all of the donors. The average percentages of the cytotoxicity of hM-CSF-untreated and hM-CSF-treated monocytes were 13 ± 7% and 27 ± 12% for K562 cells, 18 ± 9% and 31 ± 14% for U937 cells, 15 ± 9% and 27 ± 14% for Daudi cells. The cytotoxicity differences between untreated and treated monocytes were statistically significant at the level of P < 0.01. Although the average cytotoxicity against HL60 cells was very low without activation (5 ± 5%), it increased to 19 ± 11% after the preincubation with hM-CSF, which was higher than the average percentage of the baseline cytotoxicity (P < 0.01).

To investigate the role of TNF in monocyte cytotoxicity, anti-TNF antiserum was added to the preincubated monocytes before they were mixed with the target cells. As shown in Fig. 6, a neutralizing anti-TNF rabbit antiserum reduced the baseline cytotoxicity of monocytes, but the reduction was incomplete when K562, U937, and Daudi cells were used as the targets. The antibody markedly neutralized the cytotoxicity enhancement by hM-CSF, but normal rabbit serum did not exhibit any neutralizing effects.

Discussion

Monocytes preincubated with hM-CSF for 2 days killed several types of human target cells (K562, U937, Daudi, and HL60) more effectively than the control monocytes before they were mixed with the target cells. As shown in Fig. 1, a concentration-dependent cytotoxicity of monocytes, but the reduction was incomplete when K562, U937, and Daudi cells were used as the targets. The antibody markedly neutralized the cytotoxicity enhancement by hM-CSF, but normal rabbit serum did not exhibit any neutralizing effects.

The effects of polymyxin B on monocyte cytotoxicity are shown in Fig. 4. Monocytes were preincubated without (D) or with ( ) 50 ng/ml hM-CSF for 2 days, in the absence or presence of 1.0 μg/ml polymyxin B, and assayed for their cytotoxicity against K562, U937, and Daudi cells at an E:T ratio of 50:1 and against HL60 cells at an E:T ratio of 100:1. Data are shown as the mean ± SD of two separate experiments.

Discussion

Monocytes preincubated with hM-CSF for 2 days killed several types of human target cells (K562, U937, Daudi, and HL60) more effectively than the control monocytes preincubated without hM-CSF (Figs. 1 and 5). This enhancing effect was dose-dependent on the concentration of hM-CSF added to the preincubation mixture; maximal activity was observed when 50–100 ng/ml hM-CSF was added (Fig. 2). This concentration is comparable to that required to enhance the antibody-depend-
activating agents might be necessary to more effectively kill tumor targets. In fact, significant enhancement of cytotoxicity were obtained at high E:T ratios in our assay (Fig. 3).

Monocyte/macrophages can effectively kill tumor targets by means of several mechanisms. Although the detailed mechanism is not completely understood, accumulated data indicate that part of monocyte cytotoxicity can be attributed to the secretion of a cytotoxic polypeptide, TNF (21-25). Monocyte/macrophages are a major source of TNF (21), and hM-CSF is known to increase monocyte secretion of TNF in collaboration with LPS and phorbol myristate acetate (12, 13). The soluble form of such a cytotoxic factor apparently does not participate in the cytotoxicity reported here, because we could not detect any cytotoxic activity in the media conditioned by hM-CSF-preincubated monocytes (data not shown). The enhanced activity of hM-CSF-preincubated monocytes was partially inhibited by anti-TNF antiserum (Fig. 6), suggesting that TNF bound to the monocyte membrane might participate in the monocyte tumor-killing mechanism mediated by hM-CSF. However, a large amount of antiserum (5 x 10^4 neutralizing units) was needed to obtain maximal inhibition. This might be explained by the observation that an antibody generated against a recombinant form could not efficiently recognize a natural form of TNF (26). Further investigations will be required to clarify the detailed mechanism of the enhanced cytotoxicity reported here.

Human M-CSF stimulates the proliferation and growth of the human monocyte lineage as well as monocyte release of G-CSF and GM-CSF (8, 9, 11). Therefore hM-CSF may be effective in myelosuppressed patients (27). In addition, as part of its repertoire of actions, hM-CSF also stimulates several effector functions of human mature monocytes (8, 9). Recently, we observed that hM-CSF augmented the antibody-dependent tumoricidal activity of human monocytes (28); and in this paper, we have demonstrated that it also augments their antibody-independent tumoricidal activity. These results suggest that hM-CSF will be useful as part of a new clinical approach for anticancer therapy using host defense against tumors.

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REFERENCES


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Enhancing Effect of Human Monocytic Colony-stimulating Factor on Monocyte Tumoricidal Activity

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