A Macrophage Differentiating Factor Derived from Human T Cell Line HUT102 Acting on a Mouse Myeloid Cell Line M1

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KANNO, H., NOSE, M., NIKI, T., MIYAZAWA, M. and KYOGOKU, M. A Macrophage Differentiating Factor Derived from Human T Cell Line HUT102 Acting on a Mouse Myeloid Cell Line M1. Tohoku J. Exp. Med., 1993, 171(1), 43–52 —— Human T cell leukemia virus type I-transformed T cell line HUT102 constitutively secreted soluble factors which induced differentiation of a murine myeloid leukemic cell line, M1, to increase the immune complex-binding and/or phagocytizing capacity. This macrophage differentiating factor(s) (MDF) was purified from the culture supernatants of HUT102 cells by using several steps of column chromatography and novel immune-adherence and/or immune-phagocytic assays. The finally purified MDF activity was detected in the fraction that consisted of 40,000- and 45,000- molecular weight molecules. Antibodies specific for human interleukin-6 or for human granulocyte-colony stimulating factor, both of which have differentiation-inducing activity on M1 cells when used as a single factor, could not neutralize the MDF activity. These findings suggest that the 40,000- and/or 45,000- molecular weight molecules in the HUT102 cell products may be possible novel differentiation-inducing factors acting on a murine macrophage lineage across the species barrier. ——— macrophage differentiation; immune adherent assay; cytokine; HTLV-I

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Abbreviations: α-MM, α-methyl-D-mannoside; BSA, bovine serum albumin; CM, conditioned medium; DIF, macrophage differentiation inducing factor; DME, Dulbecco's modified Eagle medium; DPBS, Dulbecco's phosphate buffered saline; FCS, fetal calf serum; FPLC, Fast Protein Liquid Chromatography; G-CSF, granulocyte-colony stimulating factor; HEPES, N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid; HPLC, high pressure liquid chromatography; HTLV-I, human T cell leukemia virus type I; IL, interleukin; MDF, macrophage differentiating factor(s); MEM, Eagle's minimum essential medium; MES, 2-(N-morpholino) ethanesulfonic acid; OD, optical density; PAP, peroxidase-anti-peroxidase immune complex; PB, phosphate buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Terminal differentiation of macrophage lineage cells can be induced with various kinds of cell products. HUT102 cells (Poiesz et al. 1980), a human T cell line carrying human T cell leukemia virus type I (HTLV-I), was established from a patient with cutaneous T-cell lymphoma. HUT102 cells produce a number of soluble factors that induce differentiation of human promyelocytic cell line HL-60, such as interferon-γ, lymphotoxin, and granulocyte macrophage-colony stimulating factor (GM-CSF) (Hemmi et al. 1987, 1989). However, it remains unclear if HUT102 cells produce some other factors capable of inducing macrophage differentiation.

Most cytokines so far established exhibit a species barrier; that is, cytokines from one species do not act on target cells from another species (Nicola et al. 1985; Mosmann et al. 1987). Thus, there may be a great chance to identify new cell products by using target cells from a different species. The M1 cell line, derived from a spontaneous myeloid leukemia in an SL mouse (Ichikawa 1969; Lotem and Sachs 1974), is used to study the differentiation of cells in the murine macrophage lineage. In the present study, we detected an M1 cell differentiation-inducing activity in the HUT102 cell products. Molecular and biological characterizations suggested that this macrophage differentiating factor (MDF) may be different from the previously established differentiation-inducing cytokines.

MATERIALS AND METHODS

Cells and cell culture. M1 was a generous gift of Y. Ichikawa (Kyoto University, Kyoto). A subclone of M1 cells that showed the highest sensitivity to differentiation-inducing activities was used in the present study. M1 cells were grown in Dulbecco's modified Eagle medium (DME) (Nissui, Tokyo) supplemented with 5% heat-inactivated fetal calf serum (FCS) (M.A. Bioproducts, Walkertville, MD, USA). HUT102 cells were kindly donated by H. Hemmi (Toho University School of Medicine, Tokyo). HUT102 cells were cultured in RPMI-1640 medium (Nissui) supplemented with 5% heat-inactivated FCS, and were gradually adapted to the medium with the low concentration of FCS down to 1%. All the culture media were supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 mM N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid (HEPES). Throughout the experiments all cell lines were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air.

Antibodies to human cytokines. Rabbit anti-human interleukin-6 (IL-6) antibody (Endogen, Boston, MA, USA) and rabbit anti-human granulocyte-colony stimulating factor (G-CSF) antibody (Genzyme, Boston, MA, USA) were used to examine if they inhibit M1 cell differentiation-inducing activity of HUT102 cell products.

Determination of M1 cell differentiation-inducing activity (MDF activity). The conditioned medium (CM) of HUT102 cells and its fractions at each step of column chromatography were tested for M1 cell differentiation-inducing activity by immune-adherence and/or immune-phagocytic assays as described below. The factor(s) inducing this activity was termed as macrophage differentiating factor(s) (MDF). Each sample was dialyzed against Eagle's minimum essential medium (MEM) (Nissui) supplemented with 10 mM HEPES, at 4°C for 1 day, and was sterilized by filtration through a 0.22-μm Milipore filter (Milipore Corp., Bedford, MA, USA) before use.

M1 cells were harvested from a culture at an exponential growth phase with over 90% viability, and were resuspended at 7.5 x 10⁷ cells/ml. One hundred microliters of the M1
cell suspension was plated in a well of the V-bottomed, 96-well tissue culture plates (Nunc #249665, Nunc, Roskilde, Denmark), and a dialyzed sample (50 μl/well) was added. In the case of fractions obtained by hydroxyapatite column chromatography, a sample without dialysis (20 μl/well) was added.

After incubation at 37°C for 48 hr, the plates were centrifuged at 1,000 rpm, for 10 min, and the supernatant was aspirated. Fifty microliters of rabbit peroxidase—antiperoxidase immune complex (PAP) (DAKO, Glostrup, Denmark) diluted with DME containing 5% FCS was added to each well, and the plates were further incubated at 4°C for 1 hr. Plates were then washed twice with 150 μl/well of Dulbecco’s phosphate buffered saline (DPBS) supplemented with 0.2% (w/v) bovine serum albumin (BSA) (Fraction V, Boehringer Mannheim-Yamanouchi, Tokyo). One-hundred microliters of the substrate solution for peroxidase (the mixture of an equal volume of 0.2 mg/ml 2,2’-azino-di-[3-ethyl-benzthiazoline sulfonate] [Wako Pure Chemical, Osaka] in 100 mM citrate-phosphate buffer, pH 5.3, and 0.03% H2O2) was added to each well according to the method previously established (Miyazawa et al. 1984). After an incubation for 30 min at room temperature, the plates were centrifuged at 1,000 rpm for 10 min, and the supernatant from each well was transferred to another flat-bottomed 96-well plate. The optical density (OD) values of each supernatant were read at 405 and 510 nm in a microplate spectrophotometer (InterMed, Tokyo).

The results were represented as the difference between the OD at 405 nm and that at 510 nm as background, and the mean value was calculated from the results of triplicate wells. One unit of MDF activity was defined as the amount of activity required for 100% increase of the OD compared to wells containing untreated M1 cells.

Preparation of HUT102 serum free CM. HUT102 cells were grown in RPMI-1640 medium supplemented with 5% heat-inactivated FCS, harvested, and resuspended at 5 x 10^5 cells/ml in Daigo’s T medium (Wako Pure Chemical). After 3 days of culture, the serum-free supernatant was harvested by centrifugation (3,000 rpm, 10 min), mixed with 0.02% BSA (Sigma, St. Louis, MO, USA), and concentrated approximately 30-fold on Pellicon polysulfone membrane (Millipore) with a molecular weight exclusion limit of 10,000. The concentrated material was centrifuged at 20,000 × g, 4°C, for 1 hr to remove virus particles and cell debris, and stored at −20°C until use.

Purification of MDF

Step 1. Cation exchange chromatography. The concentrated HUT102CM was dialyzed against 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer containing 5 mM NaCl (pH 6.0), and then applied to a MonoS HR5/5 column (Pharmacia, Uppsala, Sweden) equilibrated with the same MES buffer in a Pharmacia fast protein liquid chromatography (FPLC) system. Some of the concentrated HUT102CM was applied onto a 2.6 x 36 cm column of S-Spharose Fast Flow (Pharmacia) equilibrated with the same MES buffer. After the sample was loaded, the column was washed with the MES buffer, and bound proteins were eluted discontinuously with 300 ml each of 0.25 M and 1 M NaCl in the same buffer at a flow rate of 25 ml/hr. Three fractions were collected: pass-through fraction, 0.25 M NaCl eluate, and 1 M NaCl eluate.

Step 2. Lentil lectin affinity chromatography. The fractions containing the MDF activity obtained by the cation exchange chromatography were pooled and dialyzed against DPBS containing 1 mM Ca2+ and 1 mM Mg2+ (pH 6.8). The sample was applied to a 1.0 x 11 cm column of lentil lectin-Sepharose 4B (Pharmacia) equilibrated with the same DPBS. After the sample was loaded, the column was washed with the equilibrating buffer, and bound proteins were eluted with 50 ml each of 0.15 M and 0.5 M α-methyl-D-mannoside (α-MM) in the same buffer at a flow rate of 10 ml/hr. Three fractions were collected: pass-through fraction, 0.15 M α-MM eluate, and 0.5 M α-MM eluate.

Step 3. Hydrophobic interaction chromatography. The fraction bound to the lentil lectin-affinity column and eluted with 0.15 M α-MM was dialyzed against 20 mM phosphate
buffer (PB) (pH 7.4) and applied to a 1.0 x 17 cm column of phenyl-Sepharose CL-4B (Pharmacia) equilibrated with the same PB. This column was washed with the equilibrating buffer, and bound proteins were eluted with 50 ml each of 40 and 80% (v/v) ethylene glycol in the same PB at a flow rate of 10 ml/hr. Three fractions were collected: pass-through fraction, 40% ethylene glycol eluate, and 80% ethylene glycol eluate.

**Step 4. Hydroxypatite chromatography.** The fraction passed through the hydrophobic interaction column was concentrated by ultrafiltration with a Diaflo YM-10 membrane (Amicon Corp., Lexington, MA, USA) with a molecular weight exclusion limit of 10,000, and then applied to a TSK-gel HA-1000 column (TOSOH, Tokyo) equilibrated with 10 mM PB, pH 6.8, in a FPLC system.

**Step 5. Reverse phase high pressure liquid chromatography (HPLC).** The fraction containing the MDF activity obtained through the hydroxyapatite column chromatography was concentrated by using a Speed Vac Concentrator (Savant, Farmingdale, NY, USA) and applied to a reverse phase HPLC column, PACKC8 (4.6 x 150 mm, YMC, Kyoto). The eluate was monitored by measuring the absorbance at 215 nm. Each absorbance peak fraction was collected and lyophilized. The protein from each fraction was dissolved in MEM, sterilized with 0.22-μm Milipore filter, and used in the MDF assay.

**Molecular weight determination.** Molecular weight of proteins in the fraction containing the MDF activity obtained through reverse phase HPLC was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli (Laemmli 1970). Proteins were visualized by silver staining (Morrissey 1981) with a silver stain kit (Wako Pure Chemical).

**RESULTS**

**M1 cell differentiation-inducing activity in HUT102 cell products.** Conditioned medium of HUT102 cells induced differentiation of M1 cells to increase the PAP binding capacity. Fig. 1 shows the kinetics of accumulation of M1 cell differentiation-inducing activity in culture supernatant of HUT102 cells. In the first 48 hr, M1 cell differentiation-inducing activity in the HUT102 CM increased in proportion to the incubation time, and thereafter the activity reached a plateau.

HUT102 cell products induced the differentiation of M1 cells in a dose-dependent manner (Fig. 2). However, when cycloheximide, an inhibitor of protein synthesis, was added to the culture of HUT102 cells at the concentration of 100 μg/ml, the production of M1 cell differentiation-inducing activity was remarkably inhibited (Fig. 2).

These findings showed that HUT102 cells produced a soluble factor(s) capable of inducing M1 cell differentiation. Therefore, this factor was termed as macrophage differentiating factor(s) (MDF).

**Purification of MDF from HUT102CM.** Purification of MDF in the HUT102 cell products was performed using 465 ml of the material concentrated from 10.6 liters of the pooled, serum-free, HUT102 culture supernatants. At first, 2.5 ml of concentrated material was applied to MonoS column, and then eluted with a linear gradient of NaCl from 5 to 500 mM. Higher MDF activity was observed in the fractions eluted at NaCl concentration of 0.25 M or less (results not shown). Thus, the rest of the concentrated HUT102CM was applied to a S-Sepharose Fast Flow column and eluted with 0.25 M NaCl. Since most of
Macrophage Differentiating Factor Produced by HUT102 Cells

Fig. 1. The kinetics of accumulation of M1 cell differentiation-inducing activity in the CM of HUT102 cells. HUT102 cells were resuspended at 2×10⁵ cells/ml in RPMI-1640 medium containing 1% FCS. At indicated time points, 2 ml of each supernatant was harvested sterilely, centrifuged (3,000 rpm, 10 min), and assayed for M1 cell differentiation-inducing activity. The MDF activity was measured as described under "MATERIALS AND METHODS" and represented in the value of OD₄₀₅ - OD₅₁₀.

Fig. 2. Effect of cycloheximide on the production of M1 cell differentiation-inducing activity from HUT102 cells. HUT102 cells (5×10⁵ cells/ml) were cultured in RPMI-1640 medium containing 1% FCS in the presence (■) or absence (○) of cycloheximide at a concentration of 100 μg/ml for 72 hr. The culture supernatants were then harvested by centrifugation (3,000 rpm, 10 min). To the supernatant of cycloheximide-free culture, cycloheximide was added to the same final concentration. These two samples were dialyzed, filtered, and tested in the MDF assay at the indicated concentration (from 3×2⁻ to 3×2⁷-times dilution) (see MATERIALS AND METHODS). The M1 cell differentiation-inducing activity (MDF activity) was represented in the value of OD₄₀₅ - OD₅₁₀.
previously established cytokines are glycoproteins, this MDF fraction was next applied to a lentil lectin-Sepharose column. The fraction eluted with 0.15 M $\alpha$-MM from the lentil lectin column was then applied to a phenyl-Sepharose column. The pass-through fraction from the phenyl-Sepharose step was applied to a hydroxyapatite column, and then eluted with a linear gradient of PB from 10 to 500 mM. Fractions eluted with approximately 50 to 70 mM PB showed higher MDF activity. The purification steps in column chromatography are summarized in Table 1.

The pool of active fractions on the hydroxyapatite column chromatography was applied to a C8 reverse phase column (Fig. 3). Aliquots of four major peak fractions and whole volumes of all other fractions from reverse phase column.

### Table 1. Summary of the MDF purification from HUT102CM

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Final volumes (ml)</th>
<th>Total protein (mg)</th>
<th>MDF activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrate*</td>
<td>466</td>
<td>1890</td>
<td>716,000</td>
<td>378</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>S-Sepharose</td>
<td>118</td>
<td>237</td>
<td>181,000</td>
<td>746</td>
<td>2.0</td>
<td>25.3</td>
</tr>
<tr>
<td>Lentil lectin</td>
<td>36.2</td>
<td>5.07</td>
<td>13,900</td>
<td>2740</td>
<td>7.3</td>
<td>1.94</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>17.0</td>
<td>2.45</td>
<td>6,530</td>
<td>2670</td>
<td>7.1</td>
<td>0.91</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>12.9</td>
<td>0.03</td>
<td>877</td>
<td>28300</td>
<td>74.8</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*After adding 2120 mg of BSA.

Fig. 3. Reverse phase HPLC of the MDF fraction obtained from the hydroxyapatite chromatography. Five hundred microliters of the concentrated samples was applied to a PACK C8 column and eluted with a linear gradient from 0 to 60% of acetonitrile in distilled water with 0.1% trifluoroacetic acid (-----) at a flow rate of 1 ml/min. The MDF activity of each fraction was represented in the value of $\text{OD}_{405}$-$\text{OD}_{510}$ (open bar).
Macrophage Differentiating Factor Produced by HUT102 Cells

chromatography were used in the MDF assay. One-third of the protein recovered from each of the four major peaks in reverse phase column chromatography was analyzed by SDS-PAGE (Fig. 4). MDF activity was detected only in peak 4 (Fig. 3). Fig. 4 shows that peak 4 consisted of two proteins, whose Mr were 40,000 and 45,000 (arrows). These results suggest that either of these two proteins or both are MDF.

**Effects of anti-human cytokine antibodies on MDF.** Because human IL-6 and G-CSF have been shown to induce differentiation of M1 cells (Tomida et al. 1986; Miyaura et al. 1988), anti-human IL-6 and anti-human G-CSF antibodies were employed to see if they inhibited the MDF activity in the peak fraction obtained from hydroxyapatite chromatography. Even at the highest concentration of 333 neutralizing units/ml, which should be enough to neutralize the M1 cell differentiation-inducing activities of these two cytokines (Tomida et al. 1986; Miyaura et al. 1988), neither of the antibodies had any inhibitory effects on MDF activity (results not shown). These data suggested that IL-6 and G-CSF had no relation to the MDF produced by HUT102 cells.

**DISCUSSION**

In the present study, we found that a human T cell line HUT102 produces a macrophage differentiating factor(s) acting on the mouse myeloid leukemic cell line M1 across the species barrier, which was termed MDF. Taking an advantage of this heterogeneous system, we purified this activity and found 40,000- and/or 45,000-molecular weight molecules as a possible novel factor with M1 cell differentiation-inducing activity. Our MDF assay measuring immune-adherence and/or phagocytosis of PAP using a microplate spectrophotometer was easier and more rapid than conventional phagocytic, or rosette-forming Fc receptor assays. Moreover, this system directly and quantitatively reflected the phagocytic activity and Fc receptor expression on M1 cells, because the partly purified MDF fraction obtained from hydroxyapatite chromatography induced these two activities in M1 cells in a dose-dependent manner (results not shown). Therefore, the MDF assay system used in this study may prove to be useful in screening a large number of samples for differentiation-inducing activity on macrophage cell lineage.

The finding that the MDF molecules bind to lentil lectin-affinity column (Table 1) suggests that this molecule is a glycoprotein having complex asparagine-linked oligosaccharides with bisecting L-fucose (Kornfeld et al. 1981), which is a further characteristic of some cytokines. Thus, the two bands detected in SDS-PAGE of MDF molecules purified by reverse phase HPLC may reflect the difference in glycosylation on a single protein core.

Human IL-6 (Miyaura et al. 1988) and G-CSF (Tomida et al. 1986), which also have differentiation-inducing activity across the species barrier on M1 cells that is measured by assessing the increase of phagocytic activity or Fc receptor expression, can be distinguished from the MDF in HUT102 products, because we
could not neutralize the MDF activity with anti-human IL-6 or anti-human G-CSF antibodies. Moreover, the molecular weights of human native IL-6 (Hirano et al. 1985; Shimizu et al. 1985) and G-CSF (Welte et al. 1985; Souza et al. 1986) are reported to be 21,000 and 18,000, respectively, which are quite different from that of MDF.

Recently, an M1 cell differentiation-inducing factor, macrophage differentiation inducing factor (DIF), was purified from the supernatant of human monocytic cell line THP-1 (Abe et al. 1989). This protein has N-terminal amino acid sequence highly homologous to the murine leukemia inhibitory factor. DIF also induces phagocytic activity of M1 cells, but its molecular weight (51,000) is somewhat higher than that of MDF molecules. MDF in the HUT102 cell products seems to be a possible novel differentiation-inducing factor, but further investigation of the molecular characteristics of the MDF, especially amino acid sequence analysis, is required to determine whether MDF is identical to DIF or it is an entity distinct from DIF. Production of identical or similar factors from myeloid (THP-1) and T (HUT102) cells would provide an interesting aspect of the cytokine network.

Fig. 4. SDS-PAGE of the four major peaks obtained from reverse phase HPLC. One-third of protein recovered from each peak in Fig. 3 was applied to SDS-10% PAGE in non-reduced condition. The Mr markers used were BSA (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), and soybean trypsin inhibitor (20,100). Lane a, peak 4 in Fig. 3; Lane b, peak 3 in Fig. 3; Lane c, peak 2 in Fig. 3; Lane d, peak 1 in Fig. 3.
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References


