Characterization of Macrophage Colony-Stimulating Factor in Body Fluids by Immunoblot Analysis

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We characterized the molecular species of human macrophage colony-stimulating factor (hM-CSF) found in serum and urine, using immunoblot analysis after partial purification on an antibody-bound affinity column. Although antibodies were prepared using the recombinant product of the large form of hM-CSF with a molecular weight (MW) of 85 Kd as the antigen, this immunoblot system was also capable of detecting the small form of hM-CSF with a MW of 40 to 60 Kd. A single band with a MW of 43 Kd, which reacted with anti-recombinant hM-CSF IgG but not with control IgG, was found when serum and urine from normal adults underwent electrophoresis on reduced sodium dodecyl sulfate-polyacryl-

HUMAN MACROPHAGE colony-stimulating factor (hM-CSF) is a homodimeric glycoprotein that stimulates monocyte production and activates monocyte effector functions. In addition, it seems to play an important role in placental development, and exhibits serum cholesterol-lowering activity in humans and rabbits.

hM-CSF is encoded by a single gene on chromosome 5, and alternative splicing occurring within exon 6 has been shown to generate multiple hM-CSF mRNAs in a human pancreatic carcinoma cell line, Mia Paca-2. Two different groups have isolated two different hM-CSF cDNAs, i.e., a 1.6-kilobase (kb) hM-CSF cDNA encoding a 256 amino acid polypeptide and a 4.0-kb cDNA encoding a 554 amino acid polypeptide. The polypeptide sequence deduced from the 4.0-kb clone includes the entire sequence deduced from the 1.6-kb clone and an additional sequence of 298 amino acid residues, which is inserted between residues 149 and 150 of the sequence for the smaller clone. Both polypeptides undergo signal peptide cleavage at the amino terminal and proteolytic cleavage at the carboxyl terminal, and consequently become two soluble forms. The form with an apparent molecular weight (MW) of 45 to 60 Kd is specified by the 1.6-kb cDNA and that with an apparent MW of 70 to 90 Kd is specified by the 4.0-kb cDNA. In addition to these two species, the pancreatic carcinoma cell lines PANC-1 and Mia Paca-2 secrete multiple hM-CSF species that have not yet been characterized.

Although significant molecular heterogeneity of hM-CSF has been demonstrated in an in vitro system, it is not completely certain whether multiple species are actually present or which is the dominant species in vivo. We have developed a visualization system for hM-CSF that consists of partial purification using immunoaffinity chromatography followed by immunoblot analysis of samples. Using this system, we identified the molecular species of hM-CSF protein present in human serum and urine in this study.

MATERIALS AND METHODS

Reagents. The large form of native hM-CSF (nhM-CSF) was purified from human urine, and antiserum against the large form of nhM-CSF was prepared by immunizing horses, as described previously. A recombinant product of the large form of hM-CSF (rhM-CSF) was purified to a homogeneous protein from serum-free medium conditioned by CHO-3ACSF-69 cells, as previously reported. Antiserum against the large form of rhM-CSF was prepared by immunizing rabbits with the purified material and the IgG fraction was prepared. The small form of nhM-CSF, highly purified from human urine, was kindly provided by Dr E.R. Stanley (Albert Einstein College of Medicine, Bronx, NY). The small form of rhM-CSF was purchased from Genzyme (Boston, MA). According to the manufacturer’s information, this hM-CSF was produced from a yeast expression system using a cDNA encoding the small form. Normal rabbit IgG was purchased from Organon Teknika Corp (West Chester, PA).

Human serum and urine. Serum and urine were collected from normal volunteers, patients with leukemia, and pregnant women, all of whom gave informed consent. After the removal of insoluble materials by centrifugation, the samples were stored at −20°C until use. In some experiments, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin, and 100 μg/mL phenylmethylsulfonyl fluoride (PMSF) were used to inhibit proteolysis.

Enzyme-linked immunosorbent assay (ELISA). The concentration of hM-CSF was assessed by an ELISA specific for hM-CSF that was established previously. The immunoaffinity purification. Approximately 18 mg of IgG prepared from the serum of a horse immunized with purified nhM-CSF with a MW of 85 Kd was coupled to 1 mL of formyl-cellulofine (Seikagaku Kogyo, Tokyo, Japan) according to the manufacturer’s instructions. This antibody-matrix combination was transferred to a column, washed extensively with 3.5 mol/L potassium thiocyanate.
in 0.1 mol/L sodium phosphate buffer (pH 7.0, elution buffer), and equilibrated with phosphate-buffered saline (PBS) before use. Serum and urine samples were diluted at least twofold with PBS, and sodium chloride (NaCl) was added at a final concentration of 0.3 mol/L to reduce the nonspecific background activity. Contents containing 0.1 to 1 μg of hM-CSF were added to 0.3 to 1.0 mL of a fresh batch of the antibody-matrix combination and passed through the column at least five times at a flow rate of approximately 10 mL/h, and the column was then washed with PBS containing 0.3 mol/L NaCl. To elute the hM-CSF, elution buffer was sequentially passed at 0.5 bed volumes/step through the column, each fraction was checked for the presence of hM-CSF by ELISA, and positive fractions were combined as the eluent.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.** The eluent was concentrated using an ultrafiltration membrane (UltraC3LGC; Millipore, MA) with a 10,000 MW cut-off filter, and was dissolved in Laemmli's sample buffer containing 62.5 mM/L Tris-C1 (pH 6.8), 2% SDS, 10% glycerol, and 100 mM/L dithiothreitol at a final concentration of 1 to 5 μg/mL of hM-CSF. It was then boiled for 5 minutes and subjected to electrophoresis on SDS-polyacrylamide 8% to 16% gradient gel. After electrophoresis, the gel was equilibrated for 15 minutes with blotting buffer containing 50 mM/L Tris, 380 mM/L glycine, 0.1% SDS and 20% methanol, and the proteins on the gel were transferred to a nylon membrane (Immobillon PVDF; Millipore, MA) in blotting buffer for 4 hours at 20 V using an electroblotting apparatus (FUNA-Blotting Set; Funakoshi, Tokyo, Japan). After the electrophoretic transfer, the membrane was blocked with 3% bovine serum albumin (BSA) in PBS, and probed with anti-rhM-CSF rabbit IgG or normal rabbit IgG (10 μg/mL) for 12 hours. After washing the membrane with two changes of PBS containing 0.05% Tween 20 for 15 minutes each, the membrane was further probed with ["I"]-goat antirabbit IgG, F(ab'), (New England Nuclear, Wilmington, DE) for 4 hours. After washing the membrane with three changes of PBS containing 0.05% Tween 20 for 15 minutes each, the membrane was exposed to an X-ray film and the hM-CSF band was analyzed.

**RESULTS**

The ELISA we used was based on the 'dual antibody immunometric sandwich' principle using horse and rabbit polyvalent antibodies against the large form of hM-CSF. In this assay, the hM-CSF concentration was estimated by the color development of a test sample. When the small form of hM-CSF diluted at a factor concentration suggested by the suppliers' information was assayed by this ELISA, the level of color development of small rhM-CSF and nhM-CSF was approximately 50% and 70% of that of the large form, respectively, and color development proceeded in a concentration-dependent manner (Table 1). These data showed that our ELISA system could efficiently detect the small form of hM-CSF as well as the large form.

We used this ELISA to monitor the recovery of hM-CSF from the affinity column (Table 2). When approximately 100 ng of the large and small forms of rhM-CSF were dissolved in 2 mL of fetal calf serum and applied to a 0.3 mL antibody-matrix column, both types of hM-CSF bound quantitatively to the antibody-matrix combination and were recovered in the eluted fractions with a high yield (96% and 94%, respectively). hM-CSF obtained from 20 mL of serum and 10 mL of urine that was collected from normal individuals also bound to the column and was eluted quantitatively with the percent recovery ranging from 80% to 92%. Furthermore, approximately 80 ng of the large and small forms of nhM-CSF contained in 2 mL of the passed fraction of serum were recovered in the eluted fractions with a high yield of 85% and 98%, respectively.

The initial, passed, and eluted fractions were subjected to electrophoresis using reduced SDS-PAGE and the gel was stained with Coomassie brilliant blue (Fig 1). A large amount of serum protein was removed by affinity purification and only several faint bands were seen in the lane of the eluted fraction (lane C of Fig 1), while the subunits of the small form of nhM-CSF contained in 107 ng/mL of normal serum, or 101 ng/mL of normal urine, and 106 ng/mL of normal urine, and 80 ng of the large and small forms of nhM-CSF in 2 mL of the passed fraction of normal serum were applied to a 0.3-mL antibody-matrix column. Each fraction was assayed for hM-CSF content by ELISA. The hM-CSF content in the fractions derived from serum and urine was calculated from the standard curve for the large form, and the hM-CSF content derived from the small forms of rhM-CSF and nhM-CSF was calculated from standard curves for each protein made from the data shown in Table 1.

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<th>Table 1. Detection of hM-CSF by ELISA</th>
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<td>Factor</td>
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Fig 1. SDS-PAGE analysis of a serum sample before and after affinity purification. hM-CSF was partially purified from normal serum on an affinity column, as described in Table 2. Each fraction was electrophoresed on reduced SDS-polyacrylamide gel and stained with Coomassie blue. Lane A, initial material; lane B, passed fraction; lane C, eluted fraction.

to 30 Kd (lane B), as reported previously.\(^1\) The purified small form of rhM-CSF (Genzyme) was detected as two bands at 19 Kd and 22 Kd (lane C). The MW variation observed in the small form of rhM-CSF could be explained according to the manufacturer's information as being due to differences in the glycosylation pattern.

The MW of hM-CSF present in human serum was determined by immunoblotting (Fig 3). Serum was collected from a normal adult who had a serum hM-CSF level of 5 ng/mL (lane A), a patient with chronic myelogenous leukemia who had a serum hM-CSF level of 15 ng/mL (lane B), and a pregnant woman whose serum hM-CSF level was 10 ng/mL (lane C). One major band was detected at 43 Kd in all three samples by immunoblotting of the SDS-PAGE gel run under the reducing conditions. This major band was identical in size to the subunit of the large form of hM-CSF. Several minor bands were also detected at 30, 40, 50, and 85 Kd. The 30- and 50-Kd bands might have resulted from the nonspecific binding of rabbit IgG because they were also detectable on blots performed using normal rabbit IgG instead of anti-rhM-CSF rabbit IgG as the first antibody (lanes D through F). We examined additional serum samples from five normal adults, two patients with leukocytosis who had high serum hM-CSF levels of over 10 ng/mL, and two pregnant women who also had high serum hM-CSF levels. Only a major band at 43 Kd, which reacted with anti-rhM-CSF IgG but not with normal IgG, was detected in all the samples and no other band was detected (data not shown). To exclude the possibility of proteolytic degradation of other species of hM-CSF during sample handling, several protease inhibitors were added to serum samples immediately after their preparation in some experiments. However, the same results were also obtained with these samples (data not shown).

Figure 4 shows the immunoblot analysis of urine collected from a normal adult (lane A), a patient with acute myelogenous leukemia (lane B), and a pregnant woman (lane C). A single band was detected at 43 Kd in all three cases and no other band was observed. We examined additional urine samples from eleven normal adults, seven patients with leukemia, and three pregnant women, and found that all these samples exhibited a similar pattern to that shown in Fig 4.

**DISCUSSION**

In this study, we have demonstrated that the large form of hM-CSF with a MW of 85 Kd is the dominant form in...
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Fig 4. Immunoblot analysis of hM-CSF from human urine. Urine from a normal donor (lanes A and D), a patient with leukemia (lanes B and E), and a pregnant women (lanes C and F) was analyzed by blotting the reduced SDS-PAGE gel. Each lane contained approximately 10 to 20 ng of hM-CSF. The membrane was probed with anti-rhM-CSF IgG (lanes A through C) or control IgG (lanes D through F).

Two different molecules of 40 to 70 Kd (the small form) and 70 to 90 Kd (the large form) have been examined. We only detected a 43-Kd band in urine by analysis of reduced blots (Fig 4), which corresponded to the subunit of the large form. Two bands were seen at 43 Kd and 85 Kd in equal amounts on nonreduced blots (data not shown), so the 43-Kd band on the nonreduced blot appears to represent a monomeric form of large hM-CSF because of its appearance under both reducing and nonreducing conditions. It is uncertain whether or not the monomeric protein that we detected in normal human urine was identical to the species identified by other investigators.

It has been reported that some patients with hematologic malignancy and pregnant women have elevated serum hM-CSF levels.15-18 In this study, we demonstrated that the major hM-CSF species in the circulation is the large form (Figs 3 and 4). In pregnant mice, 4.6-kb and 2.3-kb mRNAs are expressed in uterine tissues,35 which seem to correspond to the 4.0- and 1.6-kb mRNAs of hM-CSF. Therefore, we expected to detect the small form of hM-CSF in maternal samples, but we could not detect such a species in maternal serum or urine (Figs 3 and 4). There might be no enhancement or very low expression of the 1.6-kb transcript in human uterine tissues, or alternatively the factor may be produced locally and not appear in the circulation.

The small form of hM-CSF may be present in other body fluids.41 Alternatively, it may exist as a membrane-associated form in vivo and have a different role to that of the large form. Complete identification of the cellular sources of hM-CSF and molecular characterization of the translation products will enable us to better understand the physiologic significance of the different species of hM-CSF.

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