Purification and Characterization of a Cell Growth Factor from a Human Leukemia Cell Line: Immunological Identity with Ferritin

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ABSTRACT

We have succeeded in long-term cultivation of a human erythroleukemia cell line, K-562-T1 (T. Okabe, M. Fujisawa, and F. Takaku, Proc. Natl. Acad. Sci. USA, 81: 453–455, 1984). The cells grown in a protein-free chemically defined medium have been shown to produce cell growth factors (A. Mihara et al., In Vitro Cell. Dev. Biol., 23: 317–322, 1987). In this study, we have purified a cell growth factor from the conditioned medium that stimulates the proliferation of human leukemia cells, HL-60. In the purified factor, two major protein bands of 24 kDa and 22 kDa were identified on a sodium dodecyl sulfate-polyacrylamide gel. The 22 kDa protein was stained with a monoclonal antibody to the light chain of ferritin. The growth-promoting activity of the purified factor was coprecipitated with a monoclonal antibody to the light chain or heavy chain of human ferritin. These results suggest that K-562-T1 cells produce a cell growth factor that is related to ferritin.

INTRODUCTION

The ability of cancer cells to produce and respond to their own growth factor (autocrine growth factor) has become a central concept which is emerging as a unifying theme in the search for the molecular and cellular basis of malignant transformation (1). Many of malignant cells have been reported to produce growth factors in cell culture (2). The major difficulty in showing the direct evidence that the cells produce growth factors is the contamination of the growth factors by serum, which had been sequestered on the cell membrane or incorporated into cytoplasm and released into the medium used for purification of the growth factors. To exclude this troublesome possibility, we have established human cancer cell lines in protein-free chemically defined media (3, 4). The use of cells grown in a protein-free culture system provides a great advantage for the demonstration and purification of growth factors produced by the cells. K-562-T1 cells, established from an erythroleukemia cell line, K-562, have been well propagated in a protein-free chemically defined medium for 12 years. No protein supplements were used in the culture. It is therefore apparent that growth factors present in the conditioned medium of this cell line are produced by the cells themselves. The present study describes purification and characterization of a cell growth factor from the human erythroleukemia cells grown in a protein-free chemically defined medium.

MATERIALS AND METHODS

Preparation of a Serum-free Conditioned Medium

A human leukemia cell line, K-562-T1 was established from an erythroleukemia cell line, K-562, in a protein-free chemically defined medium. The cells have been grown in a protein-free synthetic medium (Dulbecco's modified Eagle's medium: Ham's F-10 nutrient mixture, 3:1, supplemented with 5.6 mm galactose, 5 mm sodium pyruvate, 0.1 mm sodium selenite, 100 units/ml penicillin G potassium, and 0.1 mg/ml streptomycin sulfate) at 37°C in a humidified 5% CO2 atmosphere. The cells have been maintained in plastic flasks (A/S Nunc, Roskilde, Denmark) with about 38 h of population-doubling time for these 12 years. The conditioned medium was harvested at a cell density of 5–10 × 10^6/ml and filtered through a GA 100 glass filter (Toyos Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was stored at −20°C until use.

Measurement of the Growth-promoting Activity

Growth-promoting activity was determined by using human myeloblastic leukemia cells, HL-60 (passage 30–50, from the American Type Culture Collection), as an indicator cell line. HL-60 cells were maintained in enriched RPMI 1640 (supplemented with 1 mm sodium pyruvate, 2 mm l-glutamine, 25 mm glucose, 0.05 mm 2-mercaptoethanol, and antibiotics) with 10% fetal calf serum (Salmon Smith Biolab Ltd., Auckland, New Zealand). For mitogenic assay, the cultured cells in exponential growth phase were washed twice in fresh enriched RPMI 1640 to remove fetal calf serum and were inoculated into 96-well flat-bottomed plates (Becton Dickinson and Co., Franklin Lakes, NJ) at the initial cell density of 1 × 10^5 cells/well in 0.1 ml of enriched RPMI 1640 without fetal calf serum. Sample (10 μl) was added to each well and the plates were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. After 5 days, the cell numbers were counted by MTr 3 assay (5). MTI dye was reduced by mitochondrial dehydrogenases to a purple formazan. The direct proportionality between dye reduction and cell number extended over a wide range of cells from 1 × 10^2 to 1 × 10^4 cells/well. Briefly, 10 μl of 0.5% MTI (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS (pH 7.4) was added to each well and the plates were incubated at 37°C for 4 h. After the addition of 100 μl of 0.04 N HCl in isopropyl alcohol to each well, the cells were vigorously mixed by pipeting. The absorbance at 570 nm (test wave length) and at 630 nm (reference wave length) was measured on an enzyme-linked immunosorbent assay ELISA microplate reader MR-700 (Dyneatech Corp., Frickenhausen, Germany). All assays were carried out in quadruplicate. One unit of growth-promoting activity was defined as the activity required for maximum stimulation of the growth of HL-60 cells (MTI absorbance was 0.5–0.6).

Purification of the Growth-promoting Activity

Ultrafiltration. All manipulations were done at 4°C, unless otherwise specified. Thirty liters of the conditioned medium were thawed and glacial acetic acid was added at a concentration of 1%. Immediately, the acidified medium was filtered through a GA 100 glass filter. Then the medium was concentrated 100-fold by using a hollow-fiber AHP 1010 (molecular weight cutoff, 50,000) (Asahi Chemical Industry, Tokyo, Japan) and dialyzed against 10 mm acetate buffer (pH 4.0) overnight. The concentrate was centrifuged at 10,000 × g for 30 min. Protein concentration was determined with a Bio-Rad protein assay kit by the method of Bradford (6) with bovine serum albumin used as a standard.

Ion-Exchange Chromatography. The dialyzed sample was applied to an SP Toyopearl cation-exchange column (1.6 × 5 cm; Tosoh Corporation, Tokyo, Japan) equilibrated with 10 mm acetate buffer, pH 4.0. The column was washed with 30 ml of the same buffer and the bound protein was eluted with a linear gradient of 0–0.8 μM NaCl in the acetate buffer. Eluted protein was detected by UV absorption at 280 nm.

Reverse-Phase High-Performance Liquid Chromatography. Active fractions from the SP Toyopearl column were loaded on an Asahipak CSP-50 reverse-phase column (1.6 × 5 cm × 10 mm; Asahi Chemical Industry) equilibrated with 0.5% acetonitrile and 99.5% water, followed by a linear gradient of 25–50% acetonitrile.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS (−), Dulbecco's phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
with 10 mM acetate buffer, pH 4.0. After a wash with 6 ml of the same buffer, the activity was eluted with a gradient of 0-40% acetonitrile in 10 mM acetate buffer, pH 4.0. Immediately after the elution, acetonitrile was removed by centrifugal concentrator.

SDS-PAGE

Active fractions obtained from the C8P-50 column were analyzed by SDS-PAGE on a 20% acrylamide gel as described by Laemmli (7) under reducing conditions. The gel was stained for protein using Coomassie Brilliant Blue R-250. Bio-Rad Low Molecular Weight SDS-PAGE Standards (Bio-Rad Laboratories, Richmond, CA) was used as a molecular mass marker. Human heart ferritin (UCB Bioproducts S. A., Allcud, Belgium) was used as a reference.

Immunoblotting

After SDS-PAGE of the active fractions from the C8P-50 column, protein was electrophoretically transferred onto a poly(vinylidene difluoride) membrane, PRO-Blott (Applied Biosystems, Foster City, CA) and immunoblotting was carried out (8, 9). A mouse monoclonal antibody to light chain of human ferritin (IgG2; Cosmo Bio Co., Ltd., Tokyo, Japan) was used as the primary antibody. The secondary antibody was alkaline phosphatase-conjugated anti-mouse IgG antibody (Tago, Inc., Burlingame, CA). Bromochloroindolyl phosphate/nitroblue tetrazolium was used as a chromogenic substrate for alkaline phosphatase. An isotype-matched mouse monoclonal antibody to β2-microglobulin (Nippon Bio-Test Laboratories, Inc., Tokyo, Japan) was used as an irrelevant reference.

Immunoprecipitation of the Activity

Immunoprecipitation of the activity was performed as described (10). The active fractions from the C8P-50 column were dialyzed against a 500-fold volume of PBS (−) overnight for neutralization. Then either a mouse monoclonal antibody to a heavy chain (RAMCO, Houston, TX) or a light chain of human ferritin (100 ng/ml) was added to each well of a 24-well flat-bottomed plate (Becton Dickinson) at 5 × 10^4 cells/well in 1 ml of serum-free enriched RPMI 1640 with the active fractions from the C8P-50 column (200 ng/ml) or human heart ferritin (100 ng/ml); 10% PBS (−), a monoclonal antibody to β2-microglobulin as a control (11). The cells were incubated and cell counts were taken every day in four wells.

Growth-promoting Activity of the Purified Factor and Ferritin on K-562-T1 Cells

K-562-T1 cells were seeded at 5 × 10^4 cells/well in 1 ml of the serum-free medium described in the preceding section. One of the following samples was added to each well: the active fractions from the C8P-50 column (200 ng/ml); human heart ferritin (100 ng/ml); 10% PBS (−), a monoclonal antibody to heavy chain of ferritin (50 µg/ml); or a monoclonal antibody to β2-microglobulin as a control (11). The cells were incubated and cell numbers were counted every day.

RESULTS

Purification of the Growth-promoting Activity. The procedure developed for the purification of the growth-promoting activity against HL-60 cells is summarized in Table 1. The activity was retained following acidification of the conditioned medium with acetic acid, while many contaminating proteins were removed as microprecipitates. The acidified concentrate of the conditioned medium was dialyzed against 10 mM acetate buffer, pH 4.0, and applied to an SP Toyopearl column. The mixture was incubated at 4°C for 2 h with vigorous shaking. Then the mixture was centrifuged at 10,000 × g for 1 min, and the residual activity in the supernatant was determined. Albumin was used to inhibit nonspecific binding of protein to Sepharose. A monoclonal antibody to β2-microglobulin was used as a reference.

Growth-promoting Activity of the Purified Factor and Ferritin on HL-60 Cells

HL-60 cells were seeded into 24-well flat-bottomed plates (Becton Dickinson) at 5 × 10^4 cells/well in 1 ml of serum-free enriched RPMI 1640 with the active fractions from the C8P-50 column (200 ng/ml) or human heart ferritin (100 ng/ml), or 10% PBS (−). The cells were incubated and cell counts were taken every day in four wells.

Immunological Characterization of the Purified Factor. Recently, we have shown that human heart ferritin which contains 24 kDa and 22 kDa (in SDS-PAGE) subunits stimulates the proliferation of HL-60 cells. We tested immunologically whether the purified factor from K562-T1 cells was related to ferritin (Fig. 3). The 22-kDa protein revealed a strong immunoreactivity with a monoclonal antibody to light chain of human ferritin, while a monoclonal antibody to

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Table 1: Purification of the growth-promoting activity on HL-60 cells

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (µg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/µg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioned medium (30 liters)</td>
<td>630,000</td>
<td>4,500</td>
<td>0.007</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Acid treatment</td>
<td>120,000</td>
<td>4,100</td>
<td>0.034</td>
<td>5</td>
<td>91</td>
</tr>
<tr>
<td>SP Toyopearl</td>
<td>1,800</td>
<td>470</td>
<td>0.26</td>
<td>37</td>
<td>10</td>
</tr>
<tr>
<td>C8P-50</td>
<td>24</td>
<td>90</td>
<td>3.8</td>
<td>542</td>
<td>2</td>
</tr>
</tbody>
</table>

Protein concentration was determined by the dye fixation method of Bradford (6). One unit of the activity was defined as the activity required for maximum stimulation of the growth of HL-60 cells.

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Fig. 1. Cation-exchange chromatography. Thirty liters of the conditioned medium were acidified, concentrated 100-fold, and dialyzed. Then the concentrate was loaded on an SP Toyopearl column equilibrated with 10 mM acetate buffer (pH 4.0). After a wash with the same buffer, the protein was eluted with a gradient of 0-0.4 M NaCl in acetate buffer. Elution of protein was monitored by absorption at 280 nm. The growth-promoting activity of each fraction was assayed with MTT.

heavy chain of human ferritin was not reactive with the 24-kDa protein. The active fractions from the C8P-50 column, however, reacted with the antibody in dot blot assay (data not shown).

The growth-promoting activity in the active fractions was coprecipitated with each of the monoclonal antibodies against ferritin (Table 2). A monoclonal antibody to \( \beta_2 \)-microglobulin, which was used for a reference, did not show any reactivity with the active fractions in either immunoblotting or immunoprecipitation.

**Growth-promoting Activity of the Purified Factor and Ferritin on HL-60 Cells.** Growth-promoting activity of the purified factor and ferritin was compared (Fig. 4). The purified factor stimulated the growth of HL-60 cells in a fashion similar to that of heart ferritin. Maximum response of HL-60 cells was obtained at 200 ng/ml of the active fractions from the C8P-50 column and at 100 ng/ml of heart ferritin.

**Growth-promoting Activity of the Purified Factor and Ferritin on K-562-T1 Cells.** To investigate the role of the purified factor and ferritin on the growth of K-562-T1 cells themselves in the protein-free culture, the active fractions from the C8P-50 column, human heart ferritin, or a monoclonal antibody against heavy chain of ferritin were added to the culture. Interestingly, both the purified factor and heart ferritin stimulated the growth of K-562-T1 cells (Fig. 5). Furthermore, an antibody to human ferritin effectively inhibited the growth of ferritin, or a monoclonal antibody against heavy chain of ferritin were added to the culture.

### Table 2 Effect of monoclonal antibodies on the growth-promoting activity in HL-60 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity in supernatant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8P-50</td>
<td>100</td>
</tr>
<tr>
<td>C8P-50 + MoAb to heavy chain of ferritin</td>
<td>11</td>
</tr>
<tr>
<td>C8P-50 + MoAb to light chain of ferritin</td>
<td>7</td>
</tr>
<tr>
<td>C8P-50 + MoAb to ( \beta_2 )-microglobulin</td>
<td>92</td>
</tr>
<tr>
<td>MoAb to heavy chain of ferritin</td>
<td>0</td>
</tr>
<tr>
<td>MoAb to light chain of ferritin</td>
<td>0</td>
</tr>
<tr>
<td>MoAb to ( \beta_2 )-microglobulin</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2. Reverse-phase high-performance liquid chromatography. Active fractions obtained from the SP Toyopearl column were applied to an Asahipak C8P-50 reverse-phase column equilibrated with 10 mM acetate buffer (pH 4.0). After a wash with the same buffer, bound protein was eluted with a gradient of 0–40% acetonitrile in the acetate buffer.

Fig. 3. Lanes A and B, SDS-PAGE on a 20% acrylamide gel under reducing condition. The gel was stained with CBB. Lane A, active fractions from the C8P-50 column. Lane B, human heart ferritin. Lanes C and D, immunoblotting of the active fractions from the C8P-50 column performed as described in "Materials and Methods." A monoclonal antibody to the light chain of ferritin was used in Lane C, and a monoclonal antibody to \( \beta_2 \)-microglobulin was used in Lane D as a reference.

Fig. 4. Growth-promoting activity of the purified factor and ferritin on HL-60 cells. HL-60 cells were seeded into 24-well flat-bottomed plates at 5 × 10^4/well in 1 ml of serum-free enriched RPMI 1640 with the active fractions from the C8P-50 column (200 ng/ml) (○), human heart ferritin (100 ng/ml) (●), or 10% PBS (○). The cell numbers were counted each day. Each point represents the mean value ± SD (bars) of four wells.

Fig. 5. Growth-promoting activity of the purified factor and ferritin on K-562-T1 cells. K-562-T1 cells were seeded at 5 × 10^4/ml in 1 ml of the serum-free medium. The active fractions from the C8P-50 column (200 ng/ml) (○), human heart ferritin (100 ng/ml) (●), 10% PBS (○), a monoclonal antibody to the heavy chain of ferritin (50 ng/ml) (△), or a monoclonal antibody to \( \beta_2 \)-microglobulin (50 μg/ml) (▲) were added to the culture.

Research.
K-562-T1 cells in the protein-free chemically defined medium. Anti-\(\beta_2\)-microglobulin antibody failed to inhibit the growth of the cells.

DISCUSSION

We have purified a cell growth factor from a human erythroleukemia cells (K-562-T1) grown in a protein-free chemically defined medium. The purified factor stimulated the growth of myeloblastic HL-60 leukemia cells. Two distinct protein bands were identified at 24 and 22 kDa on SDS-PAGE. Although a few additional bands were found on SDS gels, these bands varied with the batch of the conditioned medium used for purification. These differences may be explained by minor changes in culture conditions. Therefore, we analyzed the two protein bands of 24 and 22 kDa.

Only recently, we have found that human ferritin is capable of stimulating the growth of HL-60 cells in vitro. Ferritin is composed of two subunits, heavy chains and light chains, which migrated at 24 and 22 kDa on SDS-PAGE. These observations urged us to test whether the two protein bands derived from K-562-T1 cells are related to ferritin. The 22-kDa protein band reacted with a monoclonal antibody to light chain of human ferritin. The 24 kDa band was not stained with a monoclonal antibody to heavy chain ferritin. However, active fractions from the C8P-50 column, which contained 24 and 22 kDa proteins, reacted with the monoclonal antibody to heavy chain. It is possible that the monoclonal antibody to heavy chain ferritin is reactive with heavy chain native protein, but not with the denatured heavy chain on an SDS gel. In addition, the growth-promoting activity in the active fractions from the C8P-50 column was eliminated with each of the monoclonal antibodies.

The molecular mass and immunological characteristics suggest that this growth factor derived from K-562-T1 cells is related to ferritin. Another interesting finding of this study is that the purified factor and ferritin stimulated the growth of K-562-T1 cells. The antibody to ferritin inhibited the growth of K-562-T1 cells. These observations support the idea that K-562-T1 cells produce and respond to their own growth factor, which is related to ferritin.

REFERENCES

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