Purification and Characterization of a Human Erythrocyte-derived Growth Factor with a Wide Target Cell Spectrum*

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A cell extract from human erythrocytes promoted the growth of a wide variety of cell types, namely human and mouse myeloid cells, human and mouse T cells, human B cells, human melanoma cells, mouse transformed fibroblast cells, mouse mastocytoma cells, human lung fibroblast cells, and mouse bone marrow fibroblast/stroma-like cells. The growth-promoting activity was acid- and heat-labile and sensitive to proteases, indicating the proteinaceous nature of the molecule. The activity was also lost upon exposure to 2-mercaptoethanol.

The novel growth-promoting factor, termed basic growth factor (BGF), because of its fundamental effect and a target cell spectrum, including T cells, B cells, myeloid leukemia cells, macrophages, neutrophils, platelets, fibroblasts, and keratinocytes (1-3). Most of them have M, values below 60,000, and they work in an autocrine or paracrine manner. Many of them are specific to particular cell types and do not work across species barriers. Several factors have been identified as products of proto-oncogenes and have been implicated in transformation and tumorigenesis (4-6).

Recently, we have shown that the human monocytic cell line THP-1 produces a novel growth-promoting factor with a wide target cell spectrum, including T cells, B cells, myeloid leukemia cells, melanoma cells, and fibroblasts (7). We also observed the activity in cell extracts. During the course of the study, we noticed that extracts from various tumor cell lines contained a similar novel growth-promoting activity (8). Even erythrocytes contained the activity. In the present study, we demonstrate the purification and characterization of the factor, tentatively termed basic growth factor (BGF), from extracts of human erythrocytes because they are easily obtained in large amounts. A number of soluble factors have been shown to regulate the proliferation and/or differentiation of cells. A variety of cell types produce growth-promoting factors, including T cells, B cells, macrophages, neutrophils, platelets, fibroblasts, and keratinocytes (1-3). Most of them have M, values below 60,000, and they work in an autocrine or paracrine manner. Many of them are specific to particular cell types and do not work across species barriers. Several factors have been identified as products of proto-oncogenes and have been implicated in transformation and tumorigenesis (4-6).

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MATERIALS AND METHODS

Reagent

RPMI 1640 medium was purchased from Sigma; fetal bovine serum (FBS) was from Bocknek Laboratories (Toronto); trypsin was from Difco; o-chymotrypsin was from ICN (Cleveland, OH); Pronase E was from Kelco (San Diego, CA); proteinase K was from Merck (Darmstadt, Germany); and 2-mercaptoethanol was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human recombinant granulocyte colony-stimulating factor (G-CSF) was a gift from Chugai Pharmaceutical Co. (Tokyo).

Cell Culture

The human myelomonocytic cell line THP-1 was obtained from the American Type Culture Collection. The human promyelocytic cell line HL-60 was provided by Dr. H. Hemi (Toboku University, Sendai, Japan). The human histiocytic cell line U937, human Epstein-Barr virus-transformed B cell line Raji, human T cell line MOLT-4, mouse mastocytoma cell line P-815, and human lung fibroblast cell line TIG-1 were acquired from the Japanese Cancer Research Resources Bank (Tokyo). The human Epstein-Barr virus-transformed B cell line Daudi, human chronic myelogenous leukemia cell line K562, and mouse T cell line EL-4 were provided by Dr. T. Fujita (Fukushima Medical College, Fukushima, Japan). The human melanoma cell line A375-C6 and mouse transformed fibroblast cell line L929 were maintained in our laboratory. THP-1, HL-60, U937, K562, M1, MOLT-4, EL-4, Daudi, Raji, A375-C6, TIG-1, and L929 cells were maintained in RPMI 1640 medium containing 10% FBS and antibiotics. A375-C6 cells, TIG-1 cells, L929 cells, thymocytes, spleen cells, and bone marrow cells, cell proliferation was determined by 3H-thymidine assay (9). Proliferation of A375-C6, TIG-1, and L929 cells was determined by a dye-staining method with crystal violet (10). Proliferation was expressed as the ratio of control. Percentage of control was calculated as follows: % of control = (Atest - Acontrol) / Acontrol x 100. For thymocyte culture, concanavalin A (0.5 μg/ml) was added. Cultures were pulsed with 0.5 μCi/well of [3H]thymidine (2 Ci/mmol; bovine serum; G-CSF, granulocyte colony-stimulating factor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; IF, isoelectric focusing; IL, interleukins; TIMP-1, tissue inhibitor of metalloproteinases 1.}

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DuPont NEN) during the final 4 h of incubation and harvested, and the radioactivity incorporated into DNA was determined in a liquid scintillation counter (Alkali, Tokyo).

Stimulation of DNA Synthesis in Liquid Cultures of CSF Activity

The thymidine incorporation assay for CSF activity was performed according to the method of Gaffney et al. (11). Unfractionated bone marrow cells from ICR mice were inoculated into flat-bottomed microtiter plates at 1 x 10^6 cells/well in 200 pl of RPMI 1640 medium containing antibiotics, HEPES, and 5% FBS. Cells were cultured in the presence or absence of the purified factor, crude extracts, or human G-CSF for 5 days before labeling with [3H]thymidine (2 pcL/well) for 18 h. Radioactivity was counted in a liquid scintillation counter.

Erythrocyte Extract

Human erythrocytes were provided as "high dense erythrocytes" that were almost free of serum and platelets by blood separation solution from Aichi Prefecture Red Cross Blood Center (Seto, Japan). Cell extracts were made by hemolysis. Erythrocytes were washed twice with phosphate-buffered saline (PBS) containing 1% EDTA to remove the rest of the serum, and the erythrocyte pellet was suspended in 10 volumes of hemolysis buffer (140 m~ NH4Cl, 17 mM Tris-HCl (pH 7.65)), and incubated for 10 min at 37 °C. Then, lysates were centrifuged at 10,000 x g for 30 min at 4 °C, and supernatants were obtained as erythrocyte extracts. The extracts were filtrated using cellulose acetate membrane filters (0.8-μm pore size; ADVANTEC, Tokyo) and stored at -20 °C.

Treatment of Cell Extracts

For measurement of the heat stability of the growth-promoting activity, the cell extract was diluted 5-fold with PBS (pH 7.4) and heated to either 50 or 70 °C for 5 min. After cooling, samples were further diluted 5-fold with medium containing 5% FBS. Acid stability was assessed following 5-fold dilution of the cell extract with either 1.0 M acetic acid or 1.0 M formic acid and incubation at 4 °C for 16 h. After the addition of bovine serum albumin (BSA; final concentration of 33 μg/ml), samples were dialyzed against PBS and diluted 5-fold with medium containing 5% FBS. Susceptibility of the activity to proteases was examined by incubation of the cell extract (diluted with PBS to 50 μg of protein/ml) with an equal volume of proteases (50 μg/ml), trypsin, a-chymotrypsin, Pronase E, or proteinase K at 37 °C for 16 h. After the addition of bovine serum albumin (33 μg/ml) and incubation at 4 °C for 16 h, radioactivity was counted in a liquid scintillation counter.

Hydroxyapatite HPLC—Samples from gel filtration HPLC were applied to a 7.5 x 75-mm TSK-gel HA-1000 column (TOSOH) equilibrated with 0.01 M sodium phosphate buffer (pH 6.8). The starting buffer was 0.01 M sodium phosphate buffer (pH 6.8), and the limiting buffer was 0.5 M sodium phosphate buffer (pH 6.8). Fractions (1 ml) were collected at a flow rate of 1.0 ml/min.

Gel Filtration HPLC—1 ml of sample from anion-exchange HPLC step 2 was applied to a 7.5 mm x 60 cm TSK-gel G3000SW column (TOSOH) equilibrated with 0.01 M sodium phosphate buffer (pH 6.8). Fractions (1 ml) were collected at a flow rate of 1.0 ml/min. The column was calibrated with β-amylose (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa). It is of note that, on this column, BSA elutes at 94 kDa.

Hydroxyapatite HPLC—Samples from gel filtration HPLC were applied to a 7.5 x 75-mm TSK-gel HA-1000 column (TOSOH) equilibrated with 0.01 M sodium phosphate buffer (pH 6.8). The starting buffer was 0.01 M sodium phosphate buffer (pH 6.8), and the limiting buffer was 0.5 M sodium phosphate buffer (pH 6.8). Fractions (1 ml) were collected at a flow rate of 1.0 ml/min.

Electrophoresis

Analytical SDS-PAGE was performed according to the method of Laemmli (12) on 12.5% polyacrylamide gel (Nacalai, Minoh, Japan) using a vertical slab minigel apparatus. Molecular mass standards (Pharmacia, Uppsala) were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and a-lactalbumin (14.4 kDa).

Native gel electrophoresis was performed on Mr. 56,000, 10-cm phenyl-Toyopearl 55-500 (TOSOH). The column was washed with distilled water containing 1 M sodium phosphate buffer, 1.0 M NaCl (pH 7.4). Fractions (1 ml) were collected at a flow rate of 4.0 ml/min. The column was calibrated with myoglobin (18 kDa), carboxypeptidase A (110 kDa), bovine serum albumin (67 kDa), phospholipase A2 (75 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa). Protein bands were visualized by silver staining using a commercially available kit (Wako Pure Chemical Industries, Ltd.).

Isoelectric Focusing (IEF)

IEF was conducted by the agarose disc method as described previously (16). After focusing, samples were extracted from the agarose with distilled water and BSA for 16 h at 4 °C, and activity was measured. After addition with medium, samples were sterilized with disc filters (ADVANTEC) and then assayed for growth-promoting activity.

Amino Acid Analysis

The amino acid composition of the purified factor (0.15 μg) was determined using a sample preparation module, the Waters Picoflash™ workstation, according to the method of Bidlingmeyer et al. (16).

RESULTS

Growth-promoting Activity in Erythrocyte Extracts—A cell extract was obtained from erythrocytes to determine its growth-promoting activity on various target cells. Target cells were cultured for 3 days with varying doses of the cell extract in the presence of 5% FBS. The cell extract promoted, in a dose-dependent manner, the growth of HL-60, U937, Daudi, EL-4, M1, K562, THP-1, Raji, P-815, MOLT-4, A375-C6, TIG-1, L929, and mouse bone marrow cells, but failed to stimulate the proliferation of mouse thymocytes and spleen cells (data not shown).

The effects of various treatments on the growth-promoting activity of the cell extract were determined. The activity was partially resistant to heat treatment at 50 °C for 5 min (17% inhibition), but was lost completely when heated at 70 °C for the same period. Treatment with 1.0 μM acetic acid or formic acid completely inactivated the activity. The activity was sensitive
The erythrocyte extract was applied to a TSK-gel G3000 SW column as described under "Materials and Methods." The activity appeared at 17-19%. The fractions were subjected to reverse-phase chromatography on a Vydac C18 column. The activity appeared as a peak at 25-100 mM NaCl. The pooled active fractions were next subjected to anion-exchange chromatography on a TSK-gel HA-1000 column as described under "Materials and Methods." The growth-promoting activity was determined for HL-60 cells in each fraction.

The characteristics of this growth-promoting activity were investigated by gel filtration HPLC and IEF. This activity appeared to elute at a molecular mass of 80-120 kDa with single peaks on gel filtration HPLC (Fig. 1) and focused into a peak at pH 5.4-6.1 on IEF (Fig. 2).

The results of gel filtration column chromatography and other treatments demonstrated the protease nature of the growth-promoting activity. Pooled cell extracts were first subjected to anion-exchange chromatography on a DEAE-Toyopearl column. The major activity eluted at 25-100 mM NaCl. The active fractions were pooled and applied to the first phenyl-Toyopearl hydrophobic chromatography column. The activity appeared at a peak at 0.04-0.4 M (NH4)2SO4. The second hydrophobic chromatography step with a TSK-gel phenyl-5PW column gave the activity as a peak at 0.08-0.21 M (NH4)2SO4. The pooled active fractions from a TSK-gel HA-1000 column gave the activity as a peak between 0.08 and 0.10 M sodium phosphate buffer. Finally, the pooled active fractions were subjected to reverse-phase chromatography on a Vydac C18 column. Fig. 3 shows that the activity appeared at 17-19% CH3CN. It was of note that reverse-phase chromatography resulted in a marked decrease in specific activity. Thus, the recovery of the activity decreased to 0.003% (Table I). The purified fraction (Fraction 16) from reverse-phase column chromatography was analyzed by SDS-PAGE. On SDS-PAGE both under reducing and nonreducing conditions, the active fraction had a single band (53 kDa) (Fig. 4A). However, on native gradient PAGE, the purified factor gave a single band with a molecular mass of 270 kDa (Fig. 4B). The activity in extracts from native gradient PAGE was consistent with the band.

**Amino Acid Composition**—The NH2-terminal end of the purified factor was blocked. We then determined the amino acid composition of the factor on acid-hydrolyzed samples (Table II). The sensitivity of this factor to trypsin was assayed by the presence of lysine and arginine. It is anticipated that the factor can be cleaved by CNBr because of the presence of methionine.

**Dependence of Growth-promoting Activity on Initial Cell Density**—Serum concentrations and initial cell densities were varied to determine their effects on the growth factor activity. HL-60 cells were cultured in the presence of 1, 2, 3, 4, or 5% FBS. The stimulating activity was observed at all serum concentrations. Even without FBS, the factor stimulated proliferation, although the effect was low (data not shown). However, the activity appeared to depend on the initial cell density; namely, the stimulating activity was observed at low cell density (2.5-20 x 10^4/ml), but not at higher cell density (data not shown).

**Growth-promoting Activity of Purified Factor for Various Target Cells**—With regard to target cell spectrum, the purified factor was compared with starting extracts. Fig. 5 (A and B) shows that the growth of TIG-1, HL-60, L1299, U937, EL-4, Daudi, THP-1, A375-C6, MOLT-4, and P-815 cells was promoted by the purified factor in a dose-dependent manner. The purified factor also promoted, in a dose-dependent manner, the growth of M1, K562, and Raji cells (data not shown). The factor did not promote the thymidine incorporation of 5-day cultured mouse bone marrow cells. In contrast, the crude extract and G-CSF stimulated thymidine incorporation (data not shown).
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**Table 1**

<table>
<thead>
<tr>
<th>Purification of the growth-promoting activity in erythrocyte extracts</th>
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<td><strong>Step</strong></td>
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<tr>
<td>Cell extracts</td>
</tr>
<tr>
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<td>Phenyl-Toyopearl HPLC</td>
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<tr>
<td>Phenyl-S5cf HPLC</td>
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<td>G3000SW HPLC</td>
</tr>
<tr>
<td>HA-1000 HPLC</td>
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<tr>
<td>Reverse-phase HPLC</td>
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1 One unit of proliferation activity is defined as the reciprocal of the dilution of test samples exhibiting maximum stimulation.
2 Protein concentration was determined by a Bio-Rad kit using BSA as a standard.
3 Protein concentration was determined by absorbance at 280 nm.

**Discussion**

In this study, the erythrocyte extract appeared to contain a novel growth-promoting factor with a wide range of target cells. This activity was acid-labile and heat-inactivated when heat-treated for 5 min at 70 °C. Because of its sensitivity to proteases, the active molecule(s) is considered to be a protein. This activity was also sensitive to a reducing reagent, suggesting that intra- or intermolecular sulfide bonds or sulfhydryl residues are essential for its activity. Gel filtration column chromatography and IEF indicated that the molecular mass of this factor is 80–120 kDa and that the isoelectric point is 5.4–6.1. The molecular masses shown on SDS-PAGE and native gradient PAGE indicate that the factor consists of a homopolymer of a single polypeptide chain, not bound through an intermolecular disulfide bond. Although the molecular mass was not consistent between gel filtration HPLC and native gradient PAGE, the elution position on gel filtration chromatography is often influenced by the shape of the molecule and its affinity to the gel. Therefore, we consider that the value based on native gradient PAGE is more reliable.

About 351 μg of purified factor (BGF) was obtained from 2.9 liters of erythrocyte extract. The specific activity of the final preparation was calculated to be 346,000 units/mg of protein. However, the reverse-phase HPLC in the last step of purification, as an acid and organic solvent were used, resulted in a marked loss of specific activity of 86% in comparison with the former step. When BGF was stored at 4 or -20 °C in this solution, the activity was lost completely after 1 week. BGF, however, was active at -20 °C for at least 1 month by immediate neutralization after the reverse-phase HPLC.

Erythrocyte extracts have been used in the form of Solcoseryl (Scolo Ltd., Basel, Switzerland), which is a protein-free extract from calf blood obtained by counter-flow dialysis (17). This solution consists approximately of inorganic salts, amino acids, small peptides, purine and pyrimidine derivatives, products from carbohydrate and fat metabolism, and low molecular weight remnants of autolyzed peptides or compounds liberated by autolysis (18). Solcoseryl induces an increase in the proliferation of L929 cells (19) and the anchorage-independent growth of Syrian hamster embryo cells in primary cultures (20). But, BGF keeps the activity against dialysis, appears at M, 80,000–120,000 on gel filtration, and has a proteinaceous nature. Therefore, BGF is different or distinguishable from Solcoseryl.

Although we used erythrocytes from which most of platelets were removed, erythrocyte pellets might still have contaminated trace amounts of platelets and other blood cells. Platelets are a rich source of growth factors, including platelet-derived growth factor (21), hepatocyte growth factor (22), and transforming growth factor-β (23). However, BGF is different from platelet-derived growth factor and transforming growth factor-β with respect to isoelectric point and molecular weight and from hepatocyte growth factor with respect to the inability to bind heparin. In addition, when we separated fresh blood from a healthy volunteer into erythrocyte, leukocyte, and platelet fractions, the growth-promoting activity in the erythrocyte extracts was more than five times more potent than that in the others. As the number of erythrocytes in blood overwhelmingly surpasses that of other cells, even if the extract is contaminated with other cells, the effect can be ignored. Similarly to THP-1 cell-derived growth factor, BGF activity was dependent on an initial low cell density, not promoting the proliferation of cells cultured at an initial high cell density. Therefore, BGF appears to stimulate cell proliferation when growth conditions are not optimal.

BGF is unlikely to be a constituent of serum, such as epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor, since the activity was observed at serum concentrations from 1 to 5%. Even without serum, stimulation was observed. This laboratory as well as others reported that M1 cell growth is inhibited by interleukin (IL)-1, IL-6, and tumor necrosis factor (24, 25) and by leukemia inhibitory factor (26). Therefore, BGF is different from IL-1, IL-6, tumor necrosis factor, and leukemia inhibitory factor. This was ascertained by the observation that BGF stimulated the growth of A275-C6 cells whose growth was inhibited by IL-1, IL-6, tumor necrosis factor, and interferon-α, -β (27, 28), and -γ. In contrast to BGF, human G-CSF, macrophage colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor were unable to promote the growth of HL-60 target cells (7). In addition, BGF did not stimulate the proliferation of mouse bone marrow cells, whereas human macrophage colony-stimulating factor (29) and G-CSF can stimulate the growth of mouse bone marrow cells. These findings support the notion that macrophage colony-stimulating factor, G-CSF, granulocyte-macrophage colony-stimulating factor, and BGF are different molecular entities. Although the erythrocyte extract promoted the growth of bone marrow fibroblast/stroma-like cells, purified BGF did not. Therefore, the extract appears to contain a growth-promoting factor other than BGF. We used HL-60 cells for most of the assay during the purification because the cells are easy to manipulate. Therefore, the use of more sensitive cells such as I929 may have enabled us to detect other growth factors, although BGF still exhibited the wide target cell spectrum.

The most interesting aspect of BGF is its wide variety of target cell types. The growth of human and mouse myeloid, T, B, mastocytoma, melanoma, and transformed fibroblast cells was stimulated. Cytokine or growth factor with such activity and high molecular weight has not yet been reported. Recently, it was reported that the tissue inhibitor of metalloproteases 1...
**A**

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-94
-67
-43
-30
-20.1
-14.4

**B**

Amino acid composition of BGF

Composition is expressed as residues/mol based upon phenylthiocarbamyl-derivative analysis.

<table>
<thead>
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<th>Amino acid</th>
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<tr>
<td>Ser</td>
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<td>Arg</td>
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<td>Trp</td>
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<td>Cys</td>
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</table>

*ND, not determined.

**Table II**

**Fig. 4.** SDS-PAGE (A) and native gradient PAGE (B) of purified growth-promoting factor. Active fractions (470 ng of protein) from the Vydac C1 column were collected and analyzed on 12.5% polyacrylamide gel in the presence of 0.1% SDS under reducing (R) and nonreducing (NR) conditions (A). The purified factor(s) (620 ng) were also analyzed on 4–15% gradient polyacrylamide gel (B). The protein was detected by silver staining. The detection limit by the staining was 10 ng. Extracts were obtained from the gels run in parallel, and the growth-promoting activity was determined for HL-60 cells in each fraction.

(TIMP-1), a sialoglycoprotein with a $M_\text{r}$ of approximately 30,000 that is produced by many cell types and has been found in every human body fluid examined (30, 31), has a potent growth-promoting activity for a wide range of human and bovine cells (32). The amino acid sequence of this protein is identical to that of erythroid-potentiating activity (33, 34), which stimulates the growth of erythroid precursors (erythroid burst-forming unit and erythroid colony-forming unit) (35) and of the K562 human erythroleukemia cell line (36). TIMP-1 was different from the erythrocyte-derived factor with regard to molecular weight, and TIMP-1 was contained in FBS at a concentration of 213 ng/ml (32); thus, TIMP-1 has no effect on cells cultured in the presence of FBS. We attempted to determine the NH$_2$-terminal amino acid sequence of BGF. However, the NH$_2$-terminal end was blocked. The amino acid composition of BGF was different from those of other known growth factors, including epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, leukemia inhibitory factor, and hepatocyte growth factor, further supporting the different entities of the molecule.

The rationale of the presence of BGF in erythrocytes is interesting. Hemolysis occurs in some diseases, including thalassemia, autoimmune hemolytic anemia, and Plasmodium vivax infection, accompanying tissue injury or necrosis. BGF released...
from erythrocytes will help tissue repair alone or in cooperation with cytokines produced by other cell types.

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