ABSTRACT

We have reported previously that a factor with a molecular weight of 53,000 under SDS-polyacrylamide gel electrophoresis purified from human erythrocyte extracts promoted the growth of a wide variety of cell types from different species, including T cells, B cells, myeloid leukemia cells, melanoma cells, and mastocytoma cells, as well as normal and transformed fibroblast cells. In the present study, amino acid sequence analysis revealed that this factor has homology with human catalase. The purified factor exhibited catalase activity. Catalases derived from human erythrocytes, bovine liver, Aspergillus niger, and recombinant rat liver catalase are all able to promote the growth of cells. Antibody against human catalase absorbed both the growth-promoting activity and the enzyme activity of the purified factor. In addition, treatment of the factor with an irreversible enzyme inhibitor, aminotriazole, resulted in abrogation of both the growth-promoting activity and enzyme activity. These results indicate that the growth-promoting factor is catalase, and its activity is associated with the decomposition of hydrogen peroxide.

INTRODUCTION

Cell proliferation is regulated by a number of soluble factors. The factors, proteins or glycoproteins, produced by other cell types or by themselves, stimulate the proliferation of cells (1–3). Most of them have molecular weights below 50,000 and their effects are specific to particular cell types and do not work across species barriers. Some are present in serum, including fibroblast growth factor, epidermal growth factor, and transforming growth factor, and transferrin. Tumor cells produce a variety of growth-promoting factors that work in an autocrine manner. Structural mutations, aberrant production, or expression of the factors and their receptors are implicated in a variety of diseases.

Recently, we have shown that a human monocytic cell line, THP-1, produces a novel growth-promoting factor with a wide target cell spectrum (4). It works at low cell density. We also noticed the presence of the similar novel activities in cell extracts from various tumor cell lines (5). Even human erythrocytes contained the activity that stimulates the proliferation of human and mouse myeloid cells (HL-60, U937, K562, THP-1, and M1), human and mouse T cells (Molt-4 and EL-4), human B cells (Daudi and Raji), human melanoma cells (A375-C6), mouse transformed fibroblast cells (L929), mouse mastocytoma cells (P-815), and human lung fibroblast cells (TIG-1). The factor purified from erythrocytes did not bind heparin and has molecular weights of 53,000 and 270,000 under SDS-PAGE and native PAGE, respectively. The NH₂-terminal was blocked (6). In the present study, we demonstrated that the human erythrocyte-derived factor is catalase based on an amino acid sequence analysis, as well as enzyme studies, and the growth-promoting activity is associated with the H₂O₂ decomposing activity.

MATERIALS AND METHODS

Reagents. RPMI 1640 was purchased from Sigma Chemical Co. (St. Louis, MO); FBS from Bocknek Laboratories (Toronto, Canada); lysyl endopeptidase (Achromobacter protease I); and 3-amino-1H-1,2,4-triazole from Wako Chemicals (Osaka, Japan). Catalase human erythrocytes (29,800 catalase activity units/mg) was purchased from Athens Research and Technology, Inc. (Athens, GA); catalase from bovine liver (12,400 units/mg) from Sigma; and catalase from Aspergillus niger (1790 units/mg) from Serva Feinbiochemica GmH & Co. (Heidelberg, Germany). Recombinant rat liver catalase (23,700 units/mg) was kindly provided by Dr. T. Hashimoto (Shinshu University School of Medicine, Matsumoto, Japan). Human erythrocytes were used as "high dose erythrocyte" that were almost freed of serum and platelets from Aichi Prefecture Red Cross Blood Center (Seto, Japan).

Cell Cultures. Human promyelocytic cell line HL-60 was provided by Dr. H. Hemmi of Tohoku University (Sendai, Japan). Human chronic myelogenous leukemia cell line K562 was provided by Dr. T. Fujita of Fukushima Medical College (Fukushima, Japan). Cells were maintained in RPMI 1640 containing 100 units/ml penicillin G, 100 μg/ml streptomycin, 15 mM HEPES, and 5% heat-inactivated FBS.

Assay of Cell Proliferation. Cells suspended in RPMI 1640 supplemented with antibiotics, HEPES, 2.5% FBS, and test samples were cultured in the wells of flat-bottomed microtiter plates (100 μl of 5 × 10⁵ cells each; Falcon, Lincoln, NJ) at 37°C in air supplemented with 5% CO₂ for 3 days. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (7). Proliferation was expressed as absorbance O.D.595 and % of control. Percentage of control was calculated as:

\[
\% \text{ of control} = \frac{\text{O.D.595 in cells cultured in medium alone}}{\text{O.D.595 in cells cultured in medium with samples}} \times 100
\]

Purification of Human Erythrocyte-derived Growth Factor. A growth-promoting factor from human erythrocytes was purified as described previously (6). Briefly, the factor was purified from erythrocyte extracts by the sequential anion exchange, hydrophobic, gel filtration, hydroxylapatite, reverse--phase HPLC using a Waters Model 600E HPLC system (Waters, Milford, MA) and a Shimadzu Model LC-4A HPLC system (Shimadzu, Kyoto). The growth-promoting activity of the purified factor was 346,000 units/mg protein. It was of note, however, that the reverse-phase HPLC in the last step of purification resulted in a marked loss of specific activity by 86% in comparison with the former step.

Enzymatic Cleavage by Lysyl Endopeptidase. Enzyme digestion of the factor on PVDF membrane was carried out by the method of Iwamatsu (8). The purified factor (200 pmol) was precipitated by 20% trichloroacetic acid. After solubilizing the precipitation with gel electrophoresis buffer, the sample was electrophoresed with SDS-PAGE, and then it was transferred into PVDF membrane (Immobilon; Millipore). The protein was stained with Ponceau S. The protein was reduced at 60°C for 1 h in 200 μl of 0.5 mol guanidine containing 0.5 M Tris-HCl (pH 8.6), 0.3% EDTA, 2% acetonitrile, and 1 mg DTT. Lidoacetic acid (2.4 mg) was then dissolved with 20 μl of water, added to the reducing solution, and incubated for 20 min in the dark. The membrane was washed several times with 2% acetonitrile, followed by staining in 0.1% SDS for 5 min at 37°C. S-Carboxymethylated PVDF-blotted protein was vortexed for 20 min at room temperature in 200 μl of 0.5% PV-40 in 0.1 mm acetic acid. The membrane was cut after thorough washing with a digestion buffer [90 mm Tris-HCl (pH 9.0) and 8% acetonitrile] and then the piece was transferred into 60 μl of the digestion buffer. To this solution, Achromobacter protease I (lysyl endopeptidase) was added to become enzyme:substrate ratio of 1:100 (mol/mol) and incubated at 30°C for 18 h.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TFA, trifluoroacetic acid.
CATALASE AS A GROWTH-PROMOTING FACTOR

Elution of Peptides from Membrane and Preparation for Reverse-phase HPLC. After digestion, the reaction was terminated by the addition of 4 µl of 10% TFA. The peptides were separated by reverse-phase HPLC using a µRPC C2/C18PC3.2/3 column (Pharmacia). The starting buffer (A) was 0.1% TFA in water, and the limiting buffer (B) was 0.1% TFA in acetonitrile. The gradient was run from 0 to 40% buffer B within 30 min. Flow rate was 300 µl/min.

Amino Acid Sequence Analysis. The amino acid sequence analysis was conducted based on gas-phase Edman degradation using an Applied Biosystems Model 473A sequencer.

Absorption of Growth-promoting Activity with Antibody against Catalase. One hundred µl of PBS, 500 µg/ml of normal rabbit IgG, or rabbit IgG against human erythrocyte catalase (Athens Research and Technology, Inc) were incubated with 400 µl of protein A Sepharose gels at 4°C for 3 h. After washing the gels with PBS, the gels were diluted 2-fold with PBS. Samples (100 µl) were diluted with 4 volumes of RPMI 1640 containing 5% FBS. To this solution, 20 µl of protein A-Sepharose gels pretreated with PBS, normal rabbit IgG, or anti-catalase IgG were added and incubated at 4°C for 2 h. After centrifugation, the supernatants were collected, sterilized by filtration (pore size, 0.45 mm; ADVANTEC, Tokyo, Japan), and the growth-promoting activity and catalase activity were determined.

Treatment with Aminotriazole. Samples dialyzed against PBS were treated with 0.1 M aminotriazole and 10 mM H2O2 at 4°C for 3 h. After treatment, the samples were dialyzed against PBS and then sterilized by filtration.

Measurement of Catalase Activity. Catalase activity was determined by monitoring the decomposition of H2O2 in terms of the decrease in the absorbance at 240 nm (9). The molecular coefficient of H2O2 at 240 nm was 0.04/cm·µmol. One unit of catalase activity was defined as 1 µmol of H2O2 decomposed/min at 30°C.

RESULTS

Amino Acid Sequence Analysis of Erythrocyte-derived Growth-promoting Factor. A growth-promoting factor was purified from human erythrocyte extracts. The factor was sensitive to the treatments with heat, reducing reagent, and proteases. The factor does not bind heparin and has a molecular weight of 53,000 and 270,000 under SDS-PAGE and native PAGE, respectively. NH2-terminal of the factor was blocked (6). We then digested the factor with lysyl endopeptidase. As shown in Fig. 1, the resultants products were separated into several peaks on reverse-phase HPLC. Five peptides were collected, and their amino acid sequences were determined. Seven to 15 amino acid sequences of 4 peptides, except for peptide 5, were determined. Comparison of the amino acid sequences with SWISS-PROT protein data base revealed that the purified factor coincided with the primary amino acid sequences obtained from human catalase cDNA (Table 1). It was of note that the lysine residue, which is hydrolyzed by the lysyl endopeptidase, was found in just prior to the sequenced peptides.

Catalase Activity of the Purified Factor and the Growth-promoting Activity of Catalases. Because of the sequence analysis, the purified factor was analyzed for catalase activity. On the basis of decomposing activity of hydrogen peroxide, the purified factor appeared to have catalase activity (67,400 units/mg). We then determined the growth-promoting activity of other catalases. As shown in Fig. 2, commercially available catalases from human erythrocytes, Aspergillus niger, or bovine liver and recombinant rat liver catalase are all able to stimulate the growth of K562 cells. The purified factor also promoted the growth of the cells in a similar manner as the commercially available catalase from human erythrocytes and recombinant rat liver catalase (data not shown). Although the data were not shown, the catalases also stimulated the proliferation of other cell types.

Adsorption of the Growth-promoting and Catalase Activities with Antibody against Human Catalase. In order to confirm whether the growth-promoting factor is catalase, the purified factor and other catalases were adsorbed with rabbit antibody against human catalase. As shown in Fig. 3, the growth-promoting activity as well as catalase activity (data not shown) was adsorbed with the antibody. In contrast, normal rabbit IgG did not adsorb them.

Effect of an Inhibitor of Catalase on the Growth-promoting Activity in the Purified Factor. In order to determine whether the growth-promoting activity is associated with catalase activity, the purified factor was treated with an irreversible catalase inhibitor aminotriazole, and then the growth-promoting activity was determined. As shown in Fig. 4, both the enzyme and the growth-promoting activities were markedly inhibited. The inactivation rate of catalase and growth-promoting activity was 98.6 and 98.8%, respectively.

DISCUSSION

The growth-promoting factor with a wide target cell spectrum purified from human erythrocyte extracts appeared to be catalase. The purified factor has been shown to have isoelectric point of 5.4–6.1 and molecular weight of 53, and 270, on SDS-PAGE and native PAGE, respectively (6). Therefore, the factor has been considered to be a homopolymer of a single peptide. The partial amino acid sequences of the factor completely coincided with those of human catalase. Because lysine residues were found in just prior to the sequenced peptide, the peptides did not appear to be a result of the contaminant. One major peptide was not sequenced, presumably because it is the NH2-terminal peptide. Catalases from different sources are reported to exhibit similarities in molecular weight of approximately 220,000–240,000 and subunits that consist of tetramer of 60,000 subunits (10). Based on the similarities in physicochemical properties and amino acid sequence homology, the purified factor is considered to be catalase.

The identity between the factor and catalase was also verified by determining whether catalases obtained from various sources also exhibit the growth-promoting activity. Catalases from human erythrocytes, bovine liver, Aspergillus niger, and recombinant rat liver catalase were all able to promote the proliferation of cells. Although the dose-response was different, it was probably due to different purifies of enzyme preparations. Specific enzyme activities of both bovine liver catalase and Aspergillus catalase were low compared to other catalases. The relation between the factor and catalase was also clarified by their reactivity to antibody against human catalase. The antibody adsorbed both catalase activity and the growth-promoting activity.
Table 1 Comparison of the purified factor with human catalase

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
</tr>
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<tbody>
<tr>
<td>Peptide 1</td>
<td>NADSRDPASD QMQHWKEQRA AGKADVLTG AGNHVGDKLN</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>LVQDVVPYDPE HAPFRDRERP ERVNHAGKAG AGPVEVTHD ITKYSAKVF</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>EQHETKQIA VRFSTVAVGES GSATDVRDPR GFAPVYTED GNDVLGNGT</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>PIFFIRDPIQ PSFHIHSQKR NFQTHLKDPE MVWDFSRLRP ELSHQVSLFP</td>
</tr>
<tr>
<td>Peptide 5</td>
<td>SDGQIFGHER HMMYGSHTTF KLNNANGAEV YCKHPYKTQG GIKRLNVEDE</td>
</tr>
<tr>
<td>Peptide 6</td>
<td>ARLSQEDPPY GIRDFAIA TGYKPSWITFY IQVMTQFQAR TPFNPPFDL</td>
</tr>
<tr>
<td>Peptide 7</td>
<td>KVWHERDQPL IPVGLVLNKR NVYNYFAEVE QIAFPSHMP PGIEASFQDK</td>
</tr>
<tr>
<td>Peptide 8</td>
<td>LQGRLFAVFD YHRHRGLPNY LHVPCYPVR ARVANYQDRG PMCQOOGGG</td>
</tr>
<tr>
<td>Peptide 9</td>
<td>APWYYPBNPSG ERPQPSALE HSQYGSEVTR RFWAATDDNV TVQRAFVTIN</td>
</tr>
<tr>
<td>Peptide 10</td>
<td>LNEQQRKRLC ENAIAHLKDA QIFLOKKAVK NTEVHPDYG SHIQADLOK</td>
</tr>
</tbody>
</table>

 promoting activities were inactivated. Therefore, catalase activity is the principle of the growth-promoting activity.

The finding that the factor is catalase is unexpected. It is reported that catalase stimulates cell proliferation in particular conditions. Catalase stimulated the growth of human lymphocytes in response to phorbol myristate acetate by inactivating H₂O₂ produced by phorbol myristate acetate-stimulated monocytes (12). In a serum-free condition, the growth of a T-cell clone, MLA144, was maintained in the presence of BSA. As a catalase was substitutive for BSA, BSA was thought to work as an antioxidative reagent (13). Also in a serum-free condition the human T-cell line CEM rapidly underwent apoptosis. The conditioned medium of the cells prevented the apoptosis, and the protective factor purified from the conditioned medium appeared to be catalase (14). In a hyperoxygenic condition, catalase also promotes cell proliferation. The growth of retinal pigment epithelial cells is suppressed by 20% oxygen, and superoxide dismutase and catalase relieved the suppression (15). In the present study, the growth-promoting effect of the erythrocyte-derived factor, catalase, was also observed in the absence of serum. However, the activity is low. Therefore, it is possible that the factor promotes the growth in synergy with serum-derived growth factors in the presence of serum.

activity of the purified factor. The antibody also adsorbed the activity of other catalases. This is probably due to the amino acid sequence homology between these catalases.

The factor purified from erythrocytes promoted the growth of various cell types. The recombinant rat liver catalase also promoted the growth of various cell types, including not only hematopoietic cells but also nonhematopoietic cells, indicating that the effect is not cell type specific. Therefore, the activity is unique because most of the growth factors and growth-promoting cytokines are cell type specific.

An interesting issue is whether the growth-promoting activity is associated with the hydrogen peroxide decomposing activity. The finding that the antibody adsorbed from the catalase and the growth-promoting activities does not necessarily mean that the two activities are related, although it indicates that these activities are harbored in the same molecule. To verify this point, the factor was treated with a catalase inhibitor aminotriazole that inactivates enzyme activity by irreversibly reacting with His residue of hematin in Compound I, an enzyme-peroxide derivative (11). Both the catalase and the growth-promoting activities were inactivated. Therefore, catalase activity is the principle of the growth-promoting activity.

Fig. 2. Growth-promoting activity of catalases derived from various sources. K562 cells were cultured at 37°C for 3 days with varying doses of catalases. A.N., Aspergillus niger; H.E., human erythrocyte; B.L., bovine liver; R.L., rat liver. The mean of triplicate cultures is shown. Bars, SD.
The medium we used contained up to 10% FBS, which contained by H₂O₂ under normal culture conditions at low cell density. As blank. The mean of triplicate cultures is shown.

As shown in this study, cell proliferation appeared to be influenced by H₂O₂ under normal culture conditions at low cell density. As reported in previous papers, cell proliferation was stimulated by the factor at an initial cell density lower than 2 × 10⁴/ml (6). At a higher cell density, catalase contained in the total cells may be enough to inactivate H₂O₂ in medium. Alternatively, catalases released from a small number of degenerating cells inactivate H₂O₂. Because it is thought that intracellular catalase can hardly protect cells against extracellular H₂O₂ because catalase is stored in peroxysomes (14, 16), our findings favor the latter possibility. H₂O₂ is generated in serum-free medium when exposed to fluorescence (17). Riboflavin and tryptophan, or riboflavin and tryptophan, are implicated in the formation of a lethal level of H₂O₂. However, it is our surprise that the cell proliferation is suppressed even in the routine culture condition. The medium we used contained up to 10% FBS, which contained much BSA that has been implicated in the protection against H₂O₂ (13). Therefore, even a very small amount of H₂O₂ is able to influence the cell proliferation. The observation that a very small amount of catalase, 0.1–10 ng/ml, stimulated cell proliferation supports the notion. It is also possible that catalase inactivated intracellularly generated H₂O₂.

Our findings showed that almost all the cell types are influenced by the naturally generated H₂O₂. Practically, catalase appeared to be quite useful when we conducted cell cloning by the limiting dilution method and when we regrew the frozen cells.³

Reactive oxygens are the major principals in cell injury and are recognized as mediators of apoptosis (18). Hydrogen peroxide may induce senescence of cultured cells (19). Antioxidants, including catalase, are able to inhibit apoptosis. The proto-oncogene bcl-2 inhibits apoptosis by the decreasing generation of reactive oxygens (20). As shown in this study, catalase works as an important regulatory factor for cell proliferation, even in routine culture conditions. It is reported that the level of catalase activity in tumor cells is low (21). Therefore, it is possible that extracellular catalase protects the tumor cells and favors their proliferation. Large amounts of catalase is stored in erythrocytes. Hemolysis often occurs in leukemia and diseases including thalassemia, autoimmune hemolytic anemia, and Plasmodium vivax infection, accompanying tissue injury and necrosis. Therefore, it is possible that catalase released from erythrocytes and the damaged tissue contribute to tumor growth, the prevention of tissue injury, and repair in cooperation with cytokines or growth factors produced by other cell types.

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³ K. Onozaki, unpublished observation.
A Human Erythrocyte-derived Growth-promoting Factor with a Wide Target Cell Spectrum: Identification as Catalase

Akihiko Takeuchi, Tomomi Miyamoto, Kayo Yamaji, et al.


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