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Identification of a Novel Growth-Promoting Factor with a Wide Target Cell Spectrum from Various Tumor Cells as Catalase[1]

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We have previously reported the purification from human erythrocyte extracts of a novel growth-promoting factor with a wide target cell spectrum. The factor has been identified as catalase. As cell extracts from a variety of tumor cell types exhibited both growth-promoting and catalase activities, the relationship between the two activities was examined using cell extracts from three different cell types, human myeloid cells (U937), human melanoma cells (A375-C6), and human B cells (Daudi). The growth-promoting and catalase activities were well correlated in these cell extracts. The antibody against human catalase absorbed not only catalase activity, but also the growth-promoting activity of extracts from these cell types. Treatment of the cell extracts from these cells with an irreversible catalase inhibitor, aminotriazole, abolished both the catalase and growth-promoting activities. In contrast, glutathione peroxidase (GSH-Px) activity was neither absorbed with the anti-catalase antibody, nor inhibited by aminotriazole. In addition, GSH-Px exhibited growth-promoting activity only in the presence of glutathione (GSH). These results, in conjunction with the effect of aminotriazole on the growth-promoting activity of catalase, suggest that catalase is the major growth-promoting molecule in the cell extracts, and H_2O_2-decomposing activity is important. Northern blot analysis revealed that these cells contained authentic catalase mRNA, and the mRNA level was compatible with the catalase and growth-promoting activities in the cell extracts. These results suggest that the growth-promoting activity in the tumor cell extracts is due to catalase.

Key words: catalase, growth factor, H_2O_2, reactive oxygens, tumor cells.

Cell proliferation is regulated by a number of growth factors. The factors produced by tumor cells as well as normal cells stimulate the proliferation of the cells, as well as adjacent cells, by acting in autocrine and paracrine modes (*1-3*). In addition to the growth-stimulating effect, they modulate the functions of and interactions among normal stromal cells, thus generating a microenvironment favorable for tumor growth and progression. Most of the growth factors 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 transferrin. Structural mutation and aberrant production or expression of the factors and their receptors are implicated in tumor growth and progression. Some have been identified as oncogene products or their receptors. Recently, reactive oxygens and the redox system have been shown to play important roles in the regulation of cell proliferation, as well as in signaling of growth factors and cytokines (*4*).

We have reported that human monocytic cells (THP-1) produce a novel growth-promoting factor with a wide target cell spectrum that stimulates the proliferation of human and mouse myeloid cells (HL-60, U937, K562, 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). It works at low cell density in the presence of serum (*5*). Similar activities were observed in cell extracts from various tumor cell lines (*6*). Even human erythrocytes contained similar activity (*7*). The factor purified from human erythrocytes was unexpectedly identified as catalase, based on amino acid sequence analysis as well as enzymatic studies, and the growth-promoting activity was associated with the H_2O_2-decomposing activity (*8*).

In this study, the relation was determined between the growth-promoting and catalase activities in cell extracts of three human tumor cell lines, U937, A375, and Daudi, and we demonstrated that the growth-promoting activity was due to catalase.

MATERIALS AND METHODS

Reagents—RPMI1640 and glutathione peroxidase (GSH-

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Abbreviations: ADF/TRX, adult T cell leukemia-derived factor/human thioredoxin; CuOOH, cumene hydroperoxide; FBS, fetal bovine serum; GSH, glutathione; GSH-Px, glutathione peroxidase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Px) (680 U/mg) from bovine erythrocytes were purchased from Sigma Chemical (St. Louis, MO), fetal bovine serum (FBS) from Bocknek Laboratories (Toronto, Canada), and 3-amino-1*H*-1,2,4-triazole from Wako Chemicals (Osaka). Rabbit IgG against catalase derived from human erythrocytes was purchased from Athens Research and Technology (Athens, GA). Human liver catalase cDNA was from American Type Culture Collection (Rockville, MD).

Cell Cultures—Human promyelomonocytic cell line HL-60 was provided by Dr. H. Hemmi of Tohoku University (Sendai). Human histiocytic cell line U937 and human EB-virus transformed B cell line Daudi were provided by the Japanese Cancer Research Resources Bank (Tokyo). Human melanoma cell line A375-C6 was maintained in this laboratory. These cells were maintained in RPMI1640 containing 100 units/ml of penicillin G, 100 μg/ml of streptomycin, 15 mM HEPES, and 5% heat-inactivated FBS.

Preparation of Cell Extracts—Cells were washed twice with phosphate-buffered saline (PBS; pH 7.4) containing 1 mM EDTA and then suspended to 4×10^7 cells (for U937) or 8×10^7 cells (for Daudi and A375-C6) per ml in lysate buffer (PBS consisting of 9 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml phenylmethanesulfonyl fluoride, and 1 mM EDTA). Suspensions were rotated for 2 h at 4°C. Cell lysates were then centrifuged at $27,000\times g$ for 20 min at 4°C, and dialyzed against PBS and then the supernatants were used as cell extracts.

Assay for 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, 5×10^3 cells each; Falcon, Lincoln, NJ) at 37°C in air supplemented with 5% CO_2 for 3 days. Cell proliferation was determined by MTT assay (*9*).

Absorption of the Growth-Promoting Activity with Antibody against Catalase—One hundred microliters of PBS containing various doses of normal rabbit IgG or rabbit IgG against human erythrocyte catalase were incubated with 400 μl of Protein A-Sepharose gels in PBS at 4°C for 3 h. The gels were washed with PBS, then diluted twofold with PBS. Samples (100 μl) were diluted with 4 volumes of RPMI1640 containing 5% FBS. To this solution 20 μl of Protein A-Sepharose gel pretreated with PBS, normal rabbit IgG, or anti-catalase IgG was added and the mixture was incubated at 4°C for 2 h. After centrifugation, the supernatants were collected and sterilized by filtration (pore size 0.45 μm; ADVANTEC, Tokyo), and the growth-promoting and catalase activities were determined.

Treatment with Aminotriazole—Samples dialyzed against PBS were treated with 0.1 M aminotriazole and 10 mM H_2O_2 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 H_2O_2 in terms of the decrease in the absorbance at 240 nm (*10*). The molar absorptivity of H_2O_2 at 240 nm was 0.00349 $mM^{-1}\cdot mm^{-1}$. One unit of catalase activity was defined as 1 μmol of H_2O_2 decomposed per min at 25°C.

Measurement of Glutathione Peroxidase—GSH-Px activity was measured by the modified method of Paglia and Valentine (*11*). Briefly, 0.1 ml of test sample was added to 1.4 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.01 M EDTA. The following solutions were then added: 80 μl of 5 mM NADPH, 0.2 ml of 10 mM NaN_3, and 0.1 ml of 20 mM GSH. Finally 20 μl of GSH reductase (200 U/ml) was added, and the reaction mixture was allowed to equilibrate at 37°C. The enzyme reaction was initiated by addition of 0.1 ml of 30 mM cumene hydroperoxide (CuOOH). The conversion of NADPH to NADP was followed by continuous recording of the change in absorbance of the system at 340 nm for 3 min after initiation of the reaction. One unit was defined as the number of micromoles of NADPH oxidized per minute and calculated on the basis of a molar absorbtivity for NADPH at 340 nm of 6.22×10^3 $M^{-1}\cdot cm^{-1}$.

RNA Extraction and Northern Hybridization—Total RNA was extracted according to the method of Chromczynski and Sacchi (*12*). The amount of RNA was determined spectrophotometrically. RNA samples (10 μg of total RNA/lane) were size fractionated on agarose-formaldehyde gel, and transferred to nitrocellulose filters. After prehybridization, each filter was hybridized with ^{32}P-labeled cDNA probe at 42°C for at least 14 h using the hybridization buffer, 50% formamide, 5×SSPE (1×SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 10×Denhardt's solution and 1% SDS containing 100 mg/ml salmon sperm DNA. Probes used were as follows: *Bam*H1-digested 453 bp fragment of human liver catalase cDNA; *Pst*I-digested 1.3 kb fragment of human glyceraldehydephosphate dehydrogenase (GAPDH) cDNA. The probes were labeled by random priming (Multi Prime DNA labeling Kit, Amersham, UK). After hybridization, filters were washed twice at room temperature in 2×SSC (1×SSC: 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) for 30 min and once in 0.1×SSC containing 0.1% SDS. Filters were autoradiographed using a Bio-image analyzer (Fuji BAS 2000). Experiments were conducted more than three times.

RESULTS

Growth-Promoting and Catalase Activities in Cell Extracts—Cell extracts from three cell lines, U937, A375, and Daudi, have been shown to promote the proliferation of many cell types (*6*). As a similar growth-promoting factor with a wide target cell spectrum had been purified from human erythrocytes and identified as catalase (*8*), the relation between the growth-promoting and catalase activities was examined in these cell extracts. As shown in Table I, the extract from U937 cells exhibited the most potent activities among these cell extracts. However, the catalase and the growth-promoting activities were well correlated in all three cell extracts.

Absorption of Catalase and Growth-Promoting Activities

TABLE I. **Comparison of the growth-promoting and catalase activities in cell extracts.** The growth-promoting activity for HL-60 cells and the catalase activity were determined as described in "MATERIALS AND METHODS." One unit of the growth-promoting activity was defined as the reciprocal of the dilution of samples that exhibited 50% of their maximal effect.

Cells	Growth-promoting activity (A) (units/mg protein)	Catalase activity (B) (units/mg protein)	A/B
A375	256.1	15.3	16.7
U937	1,658.8	110.4	15.0
Daudi	420.8	28.0	15.0

with Antibody against Human Catalase—To determine whether catalase and the growth-promoting activities are due to the same molecule, the cell extracts were adsorbed with rabbit antibody against human catalase. A previous study had revealed that the antibody adsorbed catalase from many species, including human erythrocytes, bovine liver, *Aspergillus niger*, and recombinant rat liver catalase (*8*). As shown in Fig. 1, when the extracts were treated with the antibody, not only catalase activity, but also the growth-promoting activity in these cell extracts was absorbed. Neither catalase nor the growth-promoting activity was absorbed with control rabbit IgG.

Effect of Treatment with a Catalase Inhibitor on Catalase and Growth-Promoting Activities—The cell extracts were treated with an irreversible catalase inhibitor, aminotriazole, and the catalase and the growth-promoting activities were determined (Fig. 2). Both activities in these cell extracts were abolished by aminotriazole.

Glutathione Peroxidase and Growth-Promoting Activity—GSH-Px activity was present in the cell extracts of U937 (0.133 U/ml), Daudi (0.154 U/ml), and A375 (0.160 U/ml). As GSH-Px is also able to decompose H_2O_2, although it requires GSH, the growth-promoting activity may be due to GSH-Px. To address this possibility, commercial bovine erythrocyte-derived GSH-Px was examined for growth-promoting activity. Although GSH-Px stimulated the proliferation of HL-60 cells, the growth-promoting activity of the GSH-Px preparation was absorbed with the antibody against human catalase (Fig. 3a), indicating that the GSH-Px preparation was contaminated with catalase. In contrast, GSH-Px activity of the enzyme preparation was neither absorbed with the antibody, nor abolished by treatment with aminotriazole (data not shown). In an attempt to determine whether H_2O_2-decomposing activity is important, cell proliferation activity was assessed in the presence of 50 μg/ml glutathione. It is of note that the medium alone contained only 1 μg/ml GSH. As shown in Fig. 3b, GSH-Px exhibited the cell proliferation activity even after adsorption with the anti-catalase antibody. Therefore, GSH-Px can exhibit growth-promoting activity only in the presence of a sufficient amount of GSH.

Expression of Catalase mRNA in the Three Cell Lines—To determine the expression level of catalase mRNA, Northern blot analysis was conducted using the probes for human catalase and GAPDH. As shown in Fig. 4, the catalase probe specifically hybridized with a 2.6 kb band, which is consistent with the length of the reported human catalase mRNA. The level of the mRNA was consistent with the catalase activity, and was highest in U937. The magnitude of the signal in A375, U937, and Daudi cells amounted to 0.2, 1.0, and 0.12, respectively.

DISCUSSION

Catalase appeared to be the entity responsible for the growth-promoting activity in the three tumor cell types.

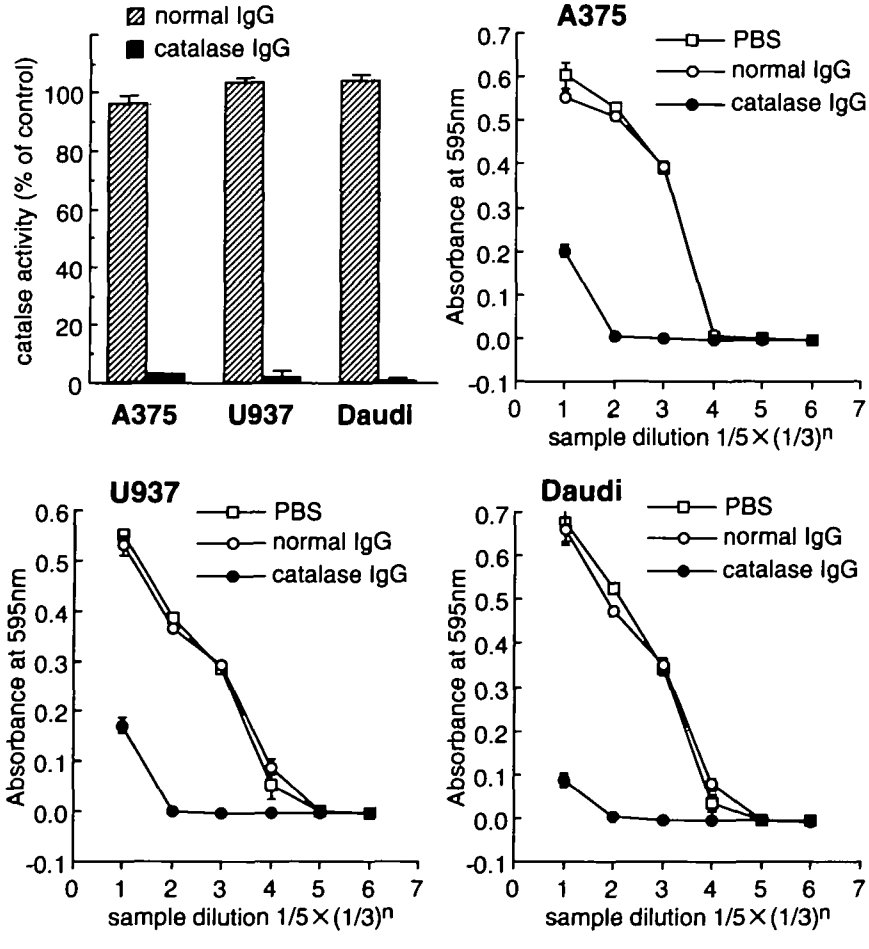

Fig. 1. **Absorption of the growth-promoting activity of cell extracts with anti-catalase antibody.** The extracts from U937, Daudi, and A375 cells were absorbed with normal rabbit IgG or rabbit IgG against human erythrocyte catalase (500 μg/ml) as described in "MATERIALS AND METHODS." After absorption, residual catalase activity and growth-promoting activity for HL-60 cells were determined. Treatment with PBS was used as the control. Mean ± SD of triplicate cultures is shown.

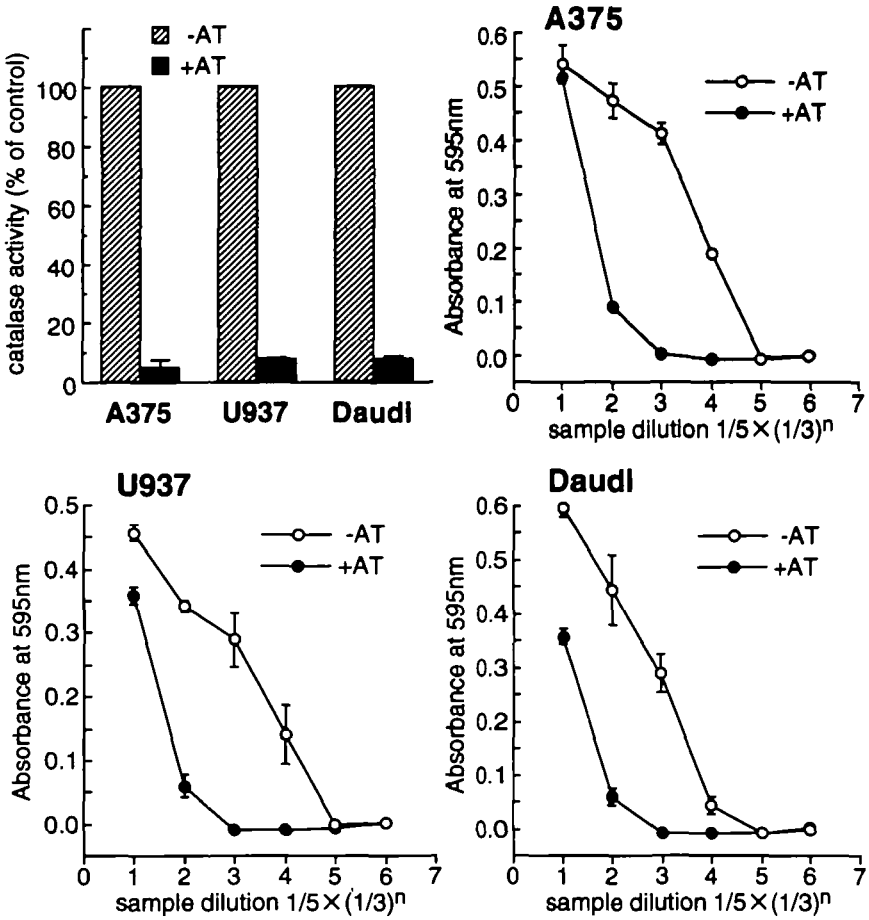

Fig. 2. **Effects of aminotriazole treatment on catalase and growth-promoting activities of cell extracts.** The cell extracts were treated with PBS (−AT) or aminotriazole (+AT) in the presence of H_2O_2 as described in "MATERIALS AND METHODS." After treatment, the samples were dialyzed against PBS, and then catalase activity was determined. Cell proliferative activity for HL-60 cells was also determined. AT added to PBS containing H_2O_2, and then treated in the same way, was used as the negative control. Mean±SD of triplicate cultures is shown.

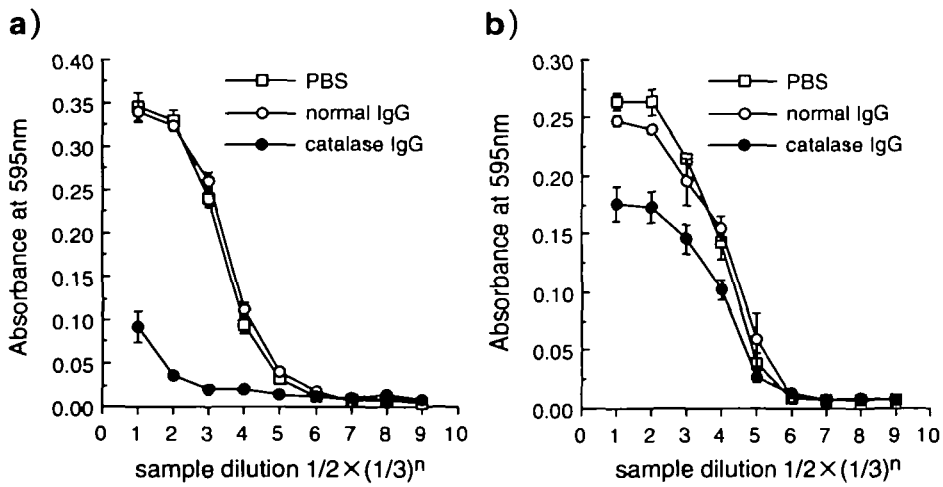

Fig. 3. **Effects of treatment with antibody against catalase and with GSH on the growth-promoting activity of GSH-Px.** Commercially available bovine GSH-Px was absorbed with normal rabbit IgG or rabbit IgG against human erythrocyte catalase as described in "MATERIALS AND METHODS." After absorption, the growth-promoting activity for HL-60 cells was determined in medium alone (a) or in the presence of GSH (50 μg/ml). Treatment with PBS was used as the control, and the sample (dilution number 1) contained 5 U/ml of GSH-Px activity and about 9 μg protein/ml. Mean±SD of triplicate cultures is shown.

Previously we have reported partial characterization of the growth-promoting activities in extracts from three cell types, U937, A375, and Daudi cells (*6*). The molecular mass of the activities located in two fractions, 100-150 and 60-70 kDa, correspond to those of the dimer and monomer of catalase, respectively. Catalase activity was also observed in the growth-promoting active fractions (Miyamoto and Onozaki, unpublished observations). Purified catalase/growth-promoting factor from human erythrocytes also eluted in the region of 80-120 kDa on gel filtration, but migrated in the 270 kDa region on native-gradient polyacrylamide gel electrophoresis (*7*). Human catalase consists of four identical subunits with 60 kDa and its isoelectric point (pI) is 5.5 (*13*). The pI values of activities in the three cell extracts were similar, 5.5-6.5. Therefore, catalase may dissociate into monomer or dimer on gel filtration. The catalase and growth-promoting activities were well correlated among these cell extracts. In addition, both the

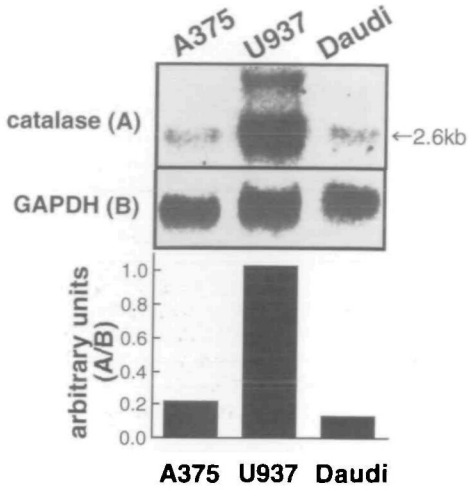

Fig. 4. **Northern blot analysis to determine catalase mRNA level in the cells.** Total RNA was extracted from A375, U937, and Daudi cells, and Northern blot analysis was conducted using probes for human catalase and GAPDH. A/B represents the relative level of catalase mRNA normalized based on the density of GAPDH.

growth-promoting and catalase activities in these three cell extracts were absorbed with the antibody against human erythrocyte catalase and were inactivated by an irreversible inhibitor specific to catalase, aminotriazole, that inhibits catalase activity by reacting with a His residue of hematin in compound I, an enzyme-peroxide derivative (*14*). The catalase activity was consistent with the level of catalase mRNA. Therefore, most, if not all, of the activities appeared to be due to catalase.

GSH-Px is also able to decompose H_2O_2, although it requires GSH for this reaction (*11*). We then determined the growth-promoting activity of commercial bovine GSH-Px. It exhibited growth-promoting activity, but the activity was absorbed with the anti-catalase antibody, and GSH-Px activity was not affected by either the antibody or aminotriazole. GSH-Px after adsorption with the antibody against catalase, however, exhibited growth-promoting activity in the presence of a sufficient amount of GSH. GSH-Px was not able to promote cell proliferation in our routine assay condition (only 1 μg/ml of GSH was present in the medium), and it requires additional GSH for its proliferative effect. The results, in conjunction with the effect of aminotriazole in the case of catalase, also suggest that H_2O_2-decomposing activity is important for their proliferative effect.

Adult T cell leukemia-derived factor (ADF)/human thioredoxin (TRX) is present in all living cells and is highly expressed in virus-infected cells (*15*). ADF/TRX is reported to stimulate cell proliferation in an autocrine manner (*16*) and to exhibit catalase-like activity as well as reducing activity (*17*). However, it requires both NADPH and thioredoxin reductase for catalase action. In addition, the molecular weight of ADF/TRX is small, 12 kDa. As shown in this study, the catalase and growth-promoting activities in U937 cell extracts were predominantly adsorbed by anti-catalase antibody and inactivated by aminotriazole. Therefore, it is unlikely that catalase/growth-promoting factor is ADF/TRX, although the remaining activity may be due to ADF/TRX.

Catalase is ubiquitous in aerobic cells from bacteria to mammalian cells (*13, 14*). In *Escherichia coli* and *Saccharomyces cerevisiae* there are two distinct catalases with only about 50% homology in nucleotide sequences (*18, 19*). In animals, only one catalase gene has been identified, peroxisomal catalase, although there are several catalases with diverse carboxyl termini (*20, 21*). Therefore, in an attempt to determine whether these cells may have a unique catalase, we conducted Northern blot analysis. All the cells expressed mRNA with the same size, and the expression levels were consistent with the catalase activity in the cell extracts. Under a non-stringent condition which allowed us to detect mRNA with 50% homology, we obtained similar results (data not shown). Therefore, it seems unlikely that a novel catalase is expressed in these cells.

The finding that the growth-promoting factor is catalase is unexpected. However, reactive oxygens are major principles in cell injury; they are recognized as mediators of growth factor or apoptosis and may induce senescence of cultured cells (*22-24*). It has been reported that catalase stimulates cell proliferation under certain conditions. Catalase stimulates the growth of human lymphocytes in response to phorbol myristate acetate (PMA) and retinal pigment epithelial cells cultured under a hyperoxygenic condition (*25, 26*). Catalase also prevents the apoptosis of neuronal cells caused by amyloid β protein deposition, which is thought to be related to Alzheimer's disease (*27*). Nevertheless, our studies revealed that catalase at very low concentration, equivalent to that of cytokines and growth factors, stimulates the proliferation of almost all cell types under general culture conditions when the cell density is low. In accord with our findings, it has been reported that a small amount of catalase released from human T cells (CEM) is enough to prevent apoptosis (*28*). Therefore, extracellular catalase works as a growth-promoting molecule. It is known that the level of catalase in tumor cells is low (*29*), and tumor cells of dormant metastases exhibit a higher incidence of apoptosis (*30*). Therefore, it is possible that extracellular catalase released from intact or degenerated tumor cells or surrounding tissues, even at a low level, protects the tumor cells and favors their proliferation alone or in cooperation with cytokines or growth factors produced by other cell types.

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