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# Correlation between the Catalase Level in Tumor Cells and Their Sensitivity to *N*-β-Alanyl-5-*S*-Glutathionyl-3,4-Dihydroxyphenylalanine (5-S-GAD)

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***N*-β-Alanyl-5-*S*-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD) exhibits selective cytotoxicity toward certain human tumor cell lines. 5-S-GAD has been shown to release hydrogen peroxide autonomously. Hydrogen peroxide is converted to water and oxygen by catalase. The purpose of this study is to determine whether or not 5-S-GAD exhibits selective cytotoxicity toward tumor cells with low catalase levels, but not toward ones with high catalase levels. We transfected MDA-MB-435S cells, which are sensitive to 5-S-GAD, with catalase cDNA to establish high catalase producer cells, and then examined their 5-S-GAD sensitivity. Similarly, we repressed catalase expression in T47D cells, which are insensitive to 5-S-GAD, by catalase RNA interference to create low catalase producer cells, and then examined their 5-S-GAD sensitivity. We show that the overexpression of catalase made MDA-MB-435S cells insensitive to 5-S-GAD, whereas the suppression of catalase made T47D cells sensitive to 5-S-GAD. The cellular catalase level was found to be crucial for cell sensitivity to 5-S-GAD.**

**Key words: catalase, 5-S-GAD, hydrogen peroxide, selective cytotoxicity, tumor therapy.**

Abbreviations: 5-S-GAD, *N*-β-alanyl-5-*S*-glutathionyl-3,4-dihydroxyphenylalanine; RNAi, RNA interference; CAT, chloramphenicol acetyltransferase; siRNA, short interfering RNA; GFP, green fluorescent protein.

5-S-GAD was originally isolated from *Sarcophaga peregraina* (flesh fly) as a novel antibacterial substance (*1*). Besides antibacterial activity, 5-S-GAD was found to show cytotoxicity toward certain human tumor cell lines (*2*, *3*). Among 38 tumor cell lines examined *in vitro*, one melanoma (LOX-IMV1) and two breast carcinoma (MDA-MB-435S and MDA-MB-231) cell lines were sensitive to 5-S-GAD with $IC_{50}$ values of 0.5–20 μM. Moreover, the growth of tumor nodules of LOX-IMV1 and MDA-MB-435S was significantly inhibited when 5-S-GAD was administered intraperitoneally to tumor-bearing nude mice (*2*). These results indicate that 5-S-GAD is potentially useful for treating human tumors. However, it is not clear why 5-S-GAD shows selective cytotoxicity toward certain tumor cell lines.

5-S-GAD is a conjugate of glutathione and β-alanyl-L-dihydroxyphenylalanine (*1*). The catechol moiety of 5-S-GAD is assumed to be readily converted to orthoquinone, accompanied by the production of hydrogen peroxide ($H_2O_2$), which is toxic to both bacteria and mammalian cells. In fact, the antibacterial activity of 5-S-GAD is abolished in the presence of catalase (*1*), an enzyme that converts $H_2O_2$ into $H_2O$ and $O_2$, indicating that the antibacterial activity of 5-S-GAD is due to the $H_2O_2$ produced from it. On the other hand, 5-S-GAD has been shown to be a potent inhibitor of protein tyrosine kinases, including v-src, which is involved in cell proliferation and differentiation (*4*, *5*).

Recently, we demonstrated that the catalase level of T47D, a 5-S-GAD-insensitive human breast carcinoma cell line, is about 3-fold higher than that of MDA-MB-435S. Moreover, the accumulation of $H_2O_2$ in the culture medium of T47D is clearly less than that in the culture medium of MDA-MB-435S when the cells are cultured in the presence of 5-S-GAD (*6*). These results suggest that the cellular catalase level is a crucial determinant for cell sensitivity to 5-S-GAD.

In this study, we overexpressed catalase in MDA-MB-435S or repressed catalase production in T47D, and then examined their 5-S-GAD sensitivities. We found that MDA-MB-435S became resistant to 5-S-GAD when catalase was overexpressed, whereas T47D became sensitive to 5-S-GAD when catalase production was repressed. These results indicate that the selective toxicity of 5-S-GAD toward certain tumor cells depends upon the catalase level.

## MATERIALS AND METHODS

*Cells and Antibodies*—The human melanoma (LOX-IMV1) and breast carcinoma (MDA-MB-231) cell lines were supplied by the Japanese Foundation of Cancer Research. Three human breast carcinoma (MDA-MB-435S, T47D and MDA-MB-468) cell lines were purchased from the American Type Culture Collection. These cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum at 37°C under 95% air–5% $CO_2$. A polyclonal antibody against human erythrocyte catalase and antiserum against chloramphenicol acetyltransferase (CAT) were purchased from Calbiochem (San Diego, CA) and Invitrogen (Carlsbad, CA), respectively.

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*Immunoblotting*—Proteins separated by electrophoresis (*7*) were transferred electrophoretically from the gel to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was immersed in blocking solution (phosphate-buffered saline containing 5% skim milk and 0.1% Tween 20) overnight at 4°C. Then the membrane was successively treated with anti-human erythrocyte catalase antibody or anti-chloramphenicol acetyltransferase serum, followed by a horseradish peroxidase-conjugated secondary antibody. Signals were detected by ECL plus Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions.

*Construction of An Expression Vector*—A plasmid containing the complete human catalase cDNA (pCAT10) was obtained from the American Type Culture Collection (*8*). Human catalase cDNA was amplified by PCR using 5′ CTA **GCT AGC** CAC CAT GGC TGA CAG CCG GGA 3′ and 5′ GG**G GTA CC**T CAC AGA TTT GCC TTC TCC 3′ as N-terminal and C-terminal side primers, respectively. In the primer sequences, restriction enzyme sites, *Nhe*I in the N-terminal and *Kpn*I in the C-terminal primer, are in boldface, and the Kozak consensus sequence (*9*) in the N-terminal primer is underlined. The Kozak consensus sequence was inserted for proper initiation of translation. The PCR product was digested with *Nhe*I and *Kpn*I, and then cloned into the *Nhe*I- and *Kpn*I-digested pcDNA3.1(+) vector (Invitrogen). This vector includes a cytomegalovirus promoter to express the inserted gene and a neomycin resistance gene driven by the SV40 promoter to select stably transfected cells. The expression vector for CAT was obtained from Invitrogen as pcDNA3.1(+)/CAT. Before transfection, the expression vectors were linearized with *Bgl*II.

*Establishment of MDA-MB-435S Cells Overexpressing Catalase or CAT*—MDA-MB-435S cells were suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum at a density of $1 \times 10^5$ cells/ml. An aliquot of cell suspension (0.5 ml) was poured into each well of a 24-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ), and the plate was incubated overnight. Transfection was performed with FuGENE 6 transfection reagent (Roche Diagnostic Corporation, Indianapolis, IN) as recommended by the manufacturer. First, FuGENE 6 (0.6 μl) and an appropriate amount of Opti-MEMI (Invitrogen) were mixed, and then 0.2 μg of linearized vector DNA was added to prepare the transfection reagent. The final volume of the transfection reagent was 20 μl. This reagent was mixed gently and incubated at room temperature for 30 min to allow FuGENE 6-DNA complex formation. Then the reagent was added to the wells and the cells were incubated for 48 h. Following incubation, the medium was removed and replaced with the selection medium [RPMI 1640 medium supplemented with 5% fetal bovine serum and 200 μg/ml G418 (Calbiochem)]. The cells were subcultured in the selection medium every 4–5 days until G418-resistant cells grew.

*Catalase Activity Assay*—Cells were lysed with RIPA buffer (phosphate-buffered saline, 5 mM EDTA, 0.01% digitonin, 0.25% sodium deoxycholate) containing a protease inhibitor cocktail (Complete Mini EDTA-free, Roche Diagnostic Corporation). Catalase activity was determined by measuring the rate of decomposition of $H_2O_2$ as described previously (*10*). The decomposition of $H_2O_2$ was followed as the decrease in absorbance at 240 nm, and the catalase activity (unit) was determined from a standard curve obtained using bovine liver catalase (Wako Pure Chemical Industries, Osaka).

*RNA Interference (RNAi)*—RNAi was performed as described previously (*11*). Single strand RNAs (21 nucleotides each) were chemically synthesized. For the annealing of short interfering RNAs (siRNAs), 20 μM each of sense and anti-sense strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C, followed by 1 h at 37°C. The cells were suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum at a density of $2 \times 10^5$ cells/ml. The cell suspension (4 ml) was poured into a 6 cm-diameter culture dish and incubated overnight. Transfection with siRNAs was performed with Oligofectamine (Invitrogen) at a final concentration 200 nM according to the manufacturer's instructions. For this, transfection solution A was prepared by mixing 8 μl of Oligofectamine and 22 μl of Opti-MEMI; transfection solution B was prepared by adding 20 μl of the siRNA solution (20 μM) to 350 μl of Opti-MEMI. The two solutions were mixed gently and then incubated at room temperature for 20 min to prepare the transfection mix. Before transfection, the culture medium was removed from each dish and the cells were washed once with Opti-MEMI. Then 1.6 ml of Opti-MEMI was poured into each dish, and 400 μl of transfection mix was overlaid on the dish. After 4 h incubation, 1 ml of RPMI 1640 medium supplemented with 15% fetal bovine serum was added to each dish and the incubation was continued. Three days later, the cells were harvested and seeded again into a 10 cm-diameter culture dish.

*Antitumor Effect of 5-S-GAD In Vitro*—Cells were suspended to RPMI 1640 medium containing 0.5% fetal bovine serum at $1 \times 10^5$ cells/ml, and the cell suspension (100 μl) was seeded into the wells of a 96-well plate coated with collagen I. The cells were incubated overnight, and then 100 μl of RPMI 1640 medium with 0.5% fetal bovine serum containing various concentrations of 5-S-GAD was added to each well. After 24 h, cell viability was assessed by alamarBlue™ (Trek Diagnostic Systems, Cleveland, OH) assay according to the manufacturer's instructions.

## RESULTS

*Expression of Catalase in Tumor Cells*—Previously, we demonstrated that 5-S-GAD exhibits selective cytotoxicity toward certain human tumor cell lines *in vitro* (*2*). Namely, one melanoma (LOX-IMV1) and two breast carcinoma (MDA-MB-231, MDA-MB-435S) cell lines were found to be sensitive to 5-S-GAD, with $IC_{50}$ values of 0.5–20 μM. The other three breast carcinoma cell lines examined, T47D, MCF-7 and MDA-MB-468, were not sensitive, with $IC_{50}$ values above 100 μM.

The results of experiments involving MDA-MB-435S and T47D suggested that the cellular catalase level is crucial for cell sensitivity to 5-S-GAD (*6*). Therefore, we examined the expression of catalase in three 5-S-GAD-sensitive and two insensitive tumor cell lines by immuno-

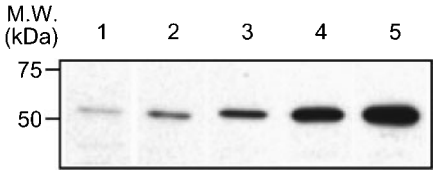


Fig. 1. **Immunoblotting of catalase in tumor cells.** Cell lysates were subjected to immunoblotting with anti-human catalase antibody. The amount of protein in each lane was 30 μg. The cell lines used were: lane 1, LOX-IMV1; lane 2, MDA-MB-231; lane 3, MDA-MB-435S; lane 4, T47D; lane 5, MDA-MB-468. The gel was calibrated with standard molecular mass markers.

blotting (Fig. 1). Judging from the intensities of the immunoblotted bands, the expression of catalase in 5-S-GAD-sensitive cells (LOX-IMV1, MDA-MB-231 and MDA-MB-435S) was clearly lower than that in insensitive cells (T47D and MDA-MB-468). The relative intensities of these bands were: 1 (LOX-IMV1), 1.5 (MDA-MB-231), 2 (MDA-MB-435S), 5 (T47D), and 10 (MDA-MB-468). Possibly, if the catalase content of tumor cells is less than 2 relative to the level in LOX-IMV1 cells, under the assay conditions employed here, the tumor cells could be expected to be sensitive to 5-S-GAD.

*Effect of Catalase Overexpression on the Sensitivity of MDA-MB-435S Cells to 5-S-GAD*—To confirm the relationship between the catalase content and 5-S-GAD sensitivity of tumor cells, we examined whether or not the overexpression of catalase in 5-S-GAD-sensitive cells would make the cells resistant to 5-S-GAD. For this, we established MDA-MB-435S cells overexpressing human catalase by transfecting cells with human catalase cDNA. As a negative control, we also established MDA-MB-435S cells expressing chloramphenicol acetyltransferase (CAT). Of the three 5-S-GAD-sensitive cell lines, MDA-MB-435S cells were most efficiently transformed with catalase cDNA. The expression of catalase in these cells was examined by immunoblotting (Fig. 2A). Increased immunoreactive catalase was detected in the transformed cells compared with the parental cells and negative control cells (CAT-expressing cells). On densitometric scanning of these bands, it was determined that the catalase level in the transformed cells was about twice that in the parent cells or negative control cells. Coincident with these facts, the catalase activity in extracts of the transformed cells was about 2-fold higher than in the other cells (Fig. 2B).

Using these cells, we investigated whether or not catalase-overexpressing cells acquire resistance to 5-S-GAD compared with parental cells or negative control cells. We treated the cells with various concentrations of 5-S-GAD for 24 h, and then compared their viability in the absence of 5-S-GAD. When the cells were treated with concentrations of 5-S-GAD below 31.25 μM, no differences in cell viability were obvious. However, in the presence of 62.5 μM 5-S-GAD, the viability of parental cells and negative control cells decreased to below 15%, whereas that of the transformed cells decreased to only about 50%. These results indicate that about a 2-fold overexpression of catalase in MDA-MB-435S cells makes them resistant to 5-S-GAD.

*Effect of Catalase RNAi on the Sensitivity of T47D Cells to 5-S-GAD*—To confirm further the relationship

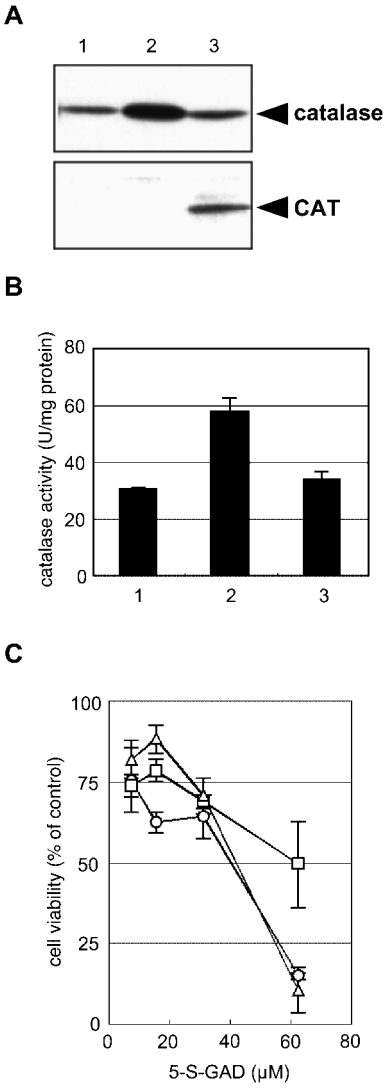


Fig. 2. **Effect of catalase overexpression in MDA-MB-435S cells on their sensitivity to 5-S-GAD.** (A) Immunoblotting of catalase in catalase-overexpressing MDA-MB-435S cells. Cell lysates were subjected to immunoblotting with anti-human catalase antibody (top) or anti-CAT antibody (bottom). The amount of protein in each lane in the top panel was 20 μg, and that in the bottom panel, 50 μg. (B) Catalase activity of catalase-overexpressing MDA-MB-435S cells. The means for two samples are shown with errors. In (A) and (B): lane 1, parental cells; lane 2, catalase-overexpressing cells; lane 3, CAT-overexpressing cells. (C) Effect of catalase overexpression on the cytotoxicity of 5-S-GAD. Cell viability relative to the control (without 5-S-GAD) was plotted against drug dose. The means of three measurements are shown with standard deviations. Circles, parental cells; squares, catalase-overexpressing cells; triangles, CAT-overexpressing cells.

between the catalase content and 5-S-GAD sensitivity of tumor cells, we tried to convert 5-S-GAD-resistant cells into sensitive cells by catalase RNAi, since it is known that chemically synthesized 19-base pair siRNA duplexes with 3′-TT overhangs promote efficient gene silencing in mammalian cells (*11*).

We first examined whether or not catalase siRNAs could inhibit the expression of catalase in T47D cells, which are known to be resistant to 5-S-GAD. For this, we

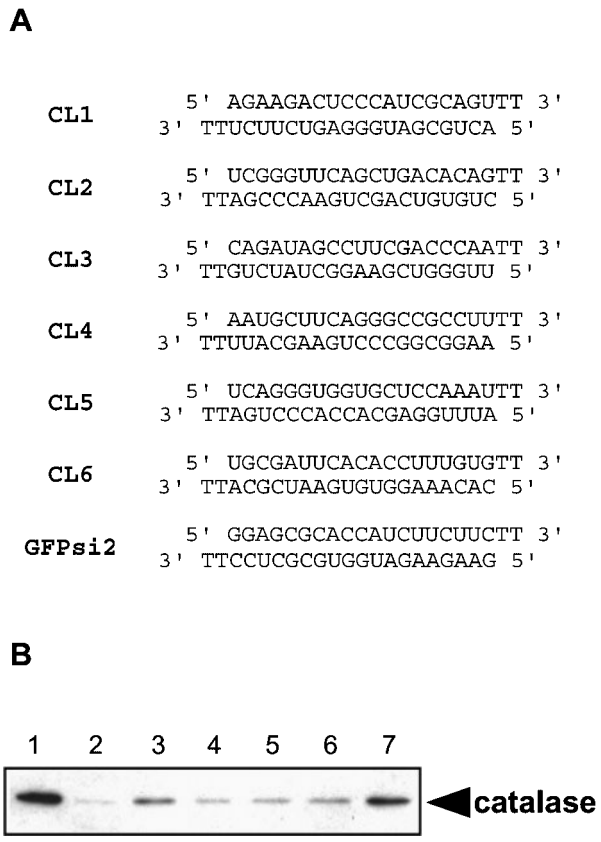


Fig. 3. **Catalase RNAi in T47D cells.** (A) Sequences of siRNAs. SiRNA sequences targeting human catalase (CL1–CL6) and green fluorescent protein (GFPsi2) (*20*) are shown. (B) T47D cells were transfected with various catalase siRNAs and the cells were harvested 5 days after transfection. Cell lysates were subjected to immunoblotting with anti-human catalase antibody. The amount of protein in each lane was 15 μg. The cells were treated as follows: lane 1, no treatment; lane 2, CL1 siRNAs; lane 3, CL2 siRNAs; lane 4, CL3 siRNAs; lane 5, CL4 siRNAs; lane 6, CL5 siRNAs; lane 7, CL6 siRNAs.

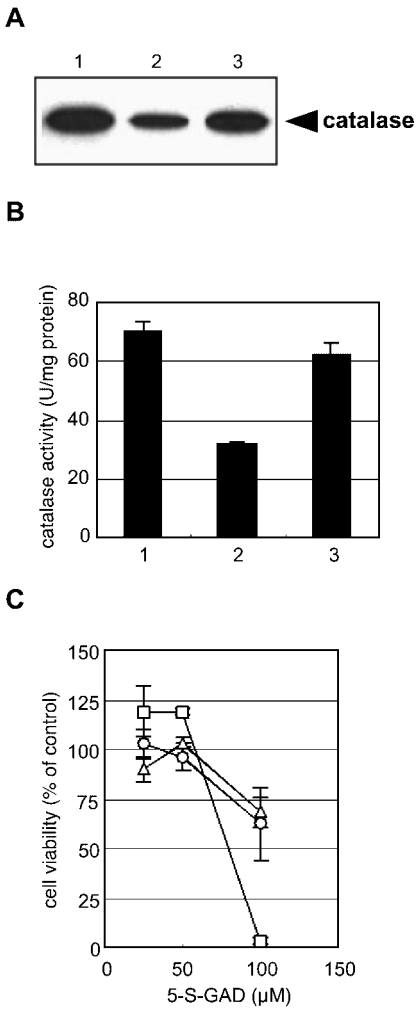


Fig. 4. **Effect of catalase RNAi on cell sensitivity to 5-S-GAD.** T47D cells were transfected with CL1 siRNAs and the cells were harvested 7 days after transfection. (A) Immunoblotting of catalase in T47D cells. Cell lysates were subjected to immunoblotting with anti-human catalase antibody. The amount of protein in each lane was 15 μg. (B) Catalase activity of catalase RNAi-treated T47D cells. The means of two measurements are shown with errors. In Fig. (A) and (B): lane 1, normal cells; lane 2, catalase RNAi-treated cells; lane 3, GFP RNAi-treated cells. (C) Effect of inhibition of catalase activity on the sensitivity to 5-S-GAD. Cell viability relative to the control (without 5-S-GAD) was plotted against drug dose. The means of three measurements are shown with standard deviations. Circles, normal cells; squares, catalase RNAi-treated cells; triangles, GFP RNAi-treated cells.

designed 6 catalase-specific siRNAs (Fig. 3A), and investigated their effect on catalase expression in T47D cells. As shown in Fig. 3B, all the catalase siRNAs inhibited catalase expression in T47D cells. We adopted CL1 because the inhibitory effect of this siRNA was most prominent.

Using CL1, we examined whether or not T47D cells become sensitive to 5-S-GAD when their catalase synthesis is inhibited by RNAi. Cellular catalase expression and cellular catalase activity decreased to 40% of the levels in normal T47D cells when the cells were cultured for 7 days after transfection with CL1. This cellular response is specific for catalase RNAi, since green fluorescent protein (GFP) RNAi (negative control) did not interfere with catalase expression (Fig. 4, A and B).

When the cells were treated with 100 μM 5-S-GAD for 24 h, the viability of normal T47D cells and negative control cells was about 65% compared with that in the absence of 5-S-GAD. On the other hand, the viability of the catalase RNAi-treated cells was almost 0% under the same conditions, indicating that the 60% inhibition of catalase expression in T47D cells by RNAi makes them sensitive to 100 μM 5-S-GAD. However, no appreciable reduction in viability was detected with 25 or 50 μM 5-S-GAD (Fig. 4C).

## DISCUSSION

In the previous study, we suggested a correlation between the catalase activity and 5-S-GAD sensitivity of human breast carcinoma cells (*6*). In this paper, we demonstrated that 5-S-GAD-sensitive cells could be converted into insensitive ones by enhancing the expression of catalase in the cells and *vice versa*. Namely, 5-S-GAD-insensitive cells could be converted into sensitive ones by

repressing the expression of catalase in the cells. Throughout these experiments, we found that the growth rates of the MDA-MB-435S and T47D cells did not change appreciably upon transfection with catalase cDNA and siRNA, respectively. Thus, it is clear that the sensitivity of these cells to 5-S-GAD is a function of their catalase level.

Previously, we demonstrated that 5-S-GAD–related compounds, such as β-alanyldopa and 5-*S*-cysteinyldopa, also exhibit selective cytotoxicity toward 5-S-GAD–sensitive cells (*2*). The cytotoxic mechanism of these compounds can be explained by $H_2O_2$, as they are expected to produce $H_2O_2$ as in the case of 5-S-GAD.

Three 5-S-GAD–sensitive tumors are so far known, and the growth of two of them (LOX-IMV1 and MDA-MB-435S) in nude mice is significantly repressed by treatment with 5-S-GAD (*2*). On the other hand, we recently found that the growth of 5-S-GAD–insensitive breast carcinomas (MDA-MB-468 and MCF-7) in nude mice was not repressed by treatment with 5-S-GAD (unpublished results). Possibly, the cellular catalase level is a crucial factor for predicting the sensitivity of tumor cells to 5-S-GAD both *in vitro* and *in vivo*. In fact, the catalase levels of three 5-S-GAD–sensitive cell lines detected on immunoblotting were significantly lower than those of two insensitive cell lines so far examined. From the results of quantitative analysis of the immunoblotting data, we concluded that the catalase level in insensitive cells is at least 2.5-fold higher than that in sensitive cells. Besides catalase, glutathione peroxidase is also known to remove $H_2O_2$. Therefore, in some tumor cells, it may be necessary to assess the contribution of glutathione peroxidase to their 5-S-GAD sensitivity.

In general, reactive oxygen species are believed to be cytotoxic toward tumor cells as well as toward normal cells. However, there have been several trials using reactive oxygen species for tumor therapy (*12–18*). In certain cancer cells, it has been reported that the levels of catalase and glutathione peroxidase are clearly lower than in normal cells (*19*). 5-S-GAD is a potentially useful chemotherapeutic agent for targeting tumors expressing low catalase levels.

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