

Induction of Cytotoxic Oxidative Stress by D-Alanine in Brain Tumor Cells Expressing *Rhodotorula gracilis* D-Amino Acid Oxidase: A Cancer Gene Therapy Strategy

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ABSTRACT

Hydrogen peroxide (H₂O₂) is a reactive oxygen species (ROS) generated in the stereoselective deamination of D-amino acids catalyzed by D-amino acid oxidase (DAAO). H₂O₂ readily crosses cellular membranes and damages DNA, proteins, and lipids. The scarcity of DAAO substrates in mammalian organisms and its co-localization with catalase in the peroxisomal matrix suggested that the cytotoxicity of ROS could be harnessed by administration of D-amino acids to tumor cells ectopically expressing DAAO in the cytoplasm. To evaluate this hypothesis, the cDNA encoding the highly active DAAO from the red yeast *Rhodotorula gracilis* was mutated to remove the carboxy-terminal peroxisomal targeting sequence. A clonal line of 9L glioma cells stably transfected with this construct (9L*daao17*) was found to synthesize active *R. gracilis* DAAO. Exposure of 9L*daao17* cells to D-alanine resulted in cytotoxicity at concentrations that were nontoxic to parental 9L cells. Depletion of cellular glutathione further sensitized 9L*daao17* cells to D-alanine (D-Ala). This result, combined with stimulation of pentose phosphate pathway activity and the production of extracellular H₂O₂ by 9L*daao17* cells incubated with D-alanine implicates oxidative stress as the mediator of cytotoxicity. These results demonstrate that expression of *R. gracilis* DAAO in tumor cells confers chemosensitivity to D-alanine that could be exploited as a novel cancer gene therapy paradigm.

OVERVIEW SUMMARY

Gene-directed enzyme prodrug therapy (GDEPT) is an antineoplastic treatment strategy designed to overcome the systemic toxicity of chemotherapy by specifically expressing a foreign enzyme in malignant cells that converts a nontoxic prodrug into a cytotoxic metabolite. The relative inefficiency of current *in situ* gene transfer methodology suggests that enzyme/prodrug combinations that produce membrane permeable metabolites will elicit a more favorable therapeutic response. Ideally, the agent produced by the transduced cell "factories" would be cytotoxic toward both proliferating and quiescent cells. We describe a novel GDEPT approach using D-amino acid oxidase from the red yeast *Rhodotorula gracilis* and D-alanine as a substrate that generates hydrogen peroxide, a reactive metabolite of oxygen that has both these characteristics. We also demonstrate

the ability to sensitize tumor cells to this GDEPT protocol by manipulating cellular antioxidant pathways.

INTRODUCTION

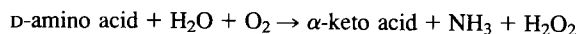
SINGLE ELECTRON REDUCTIONS of molecular oxygen give rise to a series of toxic compounds. These reactive oxygen species (ROS) are generated by ionizing radiation, drugs, and environmental pollutants, as well as by normal metabolism. Hydrogen peroxide (H₂O₂) is a ROS produced in cells by oxidases and spontaneous or enzymatic superoxide anion dismutation. H₂O₂ readily crosses cellular membranes and is known to damage biopolymers, including DNA (Beckman and Ames, 1997; Henle and Linn, 1997), proteins (Berlett and Stadtman, 1997), and lipids by direct oxidation or via transition metal-driven Haber-Weiss reduction to the extremely reactive hydroxyl rad-

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ical (Halliwell and Gutteridge, 1989). Recent evidence also indicates that H₂O₂ may induce apoptosis (Hockenbery *et al.*, 1993; Kane *et al.*, 1993; Johnson *et al.*, 1996; Simizu *et al.*, 1996; Bladier *et al.*, 1997).

Although the majority of interest in ROS has been directed to their role in pathophysiology, it is appreciated that the cytotoxicity of ROS plays a mechanistic role in a number of anti-neoplastic therapies. The most widely employed of these modalities is radiation therapy, which produces hydroxyl radicals by direct radiolytic attack on water. ROS have also been implicated as cytotoxic mediators for a number of chemotherapeutic agents including bleomycin, doxorubicin, and others. Experimental treatment modalities have been designed to produce ROS specifically in tumors. These range from photodynamic therapy (Halliwell and Gutteridge, 1989) to treatment of tumor-bearing patients or animals with preformed H₂O₂ (Makino and Tanaka, 1953; Turner, 1953; Green and Westrop, 1958; Sugara, 1958; Mealy, 1965). Investigators have also employed the ROS-generating enzymes xanthine oxidase (XO) (Yoshikawa *et al.*, 1995) and glucose oxidase (GO) as anti-cancer agents. Nathan and Cohn pioneered this approach *in vivo* when they demonstrated that GO conjugated to carboxylated latex microspheres prevented tumor cell growth in mice (Nathan and Cohn, 1981). Liposome-encapsulated glucose oxidase has also been used to eradicate experimental plasmacytomas (Samoszuk *et al.*, 1996). Previously, we have demonstrated that intratumoral H₂O₂ production following injection of polyethylene glycol (PEG)-stabilized GO into established subcutaneous rat 9L tumors resulted in a significant tumor growth delay (Ben-Yoseph and Ross, 1994). However, regulation of ROS production by exogenously administered GO in tumor-bearing hosts is problematic because the availability of its substrates, oxygen and glucose, cannot be significantly modulated. In fact, the unregulated production of ROS by intratumoral PEG-GO injections required administration of antioxidants to minimize systemic toxicity. Similarly, although XO is a lower-activity enzyme than GO, its production of superoxide cannot be regulated *in vivo* because it is a promiscuous enzyme with a wide range of ubiquitous substrates (Massey, 1973).

To overcome the limitations of GO and XO, we have proposed the use of D-amino acid oxidase (DAAO; EC 1.4.3.3) for cancer treatment (Ben-Yoseph and Ross, 1994). DAAO catalyzes the stereoselective oxidative deamination of D-amino acids via the following reaction:



The stereoselectivity of *Rhodotorula gracilis* DAAO appears to be absolute; L-amino acids are neither substrates nor inhibitors of the *R. gracilis* enzyme (Pollegioni *et al.*, 1992). Although mammalian DAAO can oxidize glycine, the only achiral amino acid, glycine is a poor substrate for the DAAO homolog from the red yeast *R. gracilis* that required 300 mM glycine to observe any enzyme-monitored activity (Pollegioni *et al.*, 1992). Thus, production of ROS by *R. gracilis* DAAO in tumor cells could be regulated by exogenous administration of D-amino acids that are rare in mammalian organisms (Konno and Yasumura, 1992). Moreover, *R. gracilis* can grow on D-Ala as a sole carbon and nitrogen source (Pilone *et al.*, 1989) and its DAAO homolog is catalytically efficient. This isoform tightly

binds its FAD co-factor (Casalin *et al.*, 1991) and has been reported to have a specific activity of 180 U/mg at 37°C with D-Ala substrate, as well as low substrate *K_M* values (submillimolar for many D-amino acids) (Pollegioni *et al.*, 1992). In contrast, mammalian DAAO homologs are comparatively inactive. For example, the *K_m* for D-Ala is 6.5 mM for hog kidney DAAO, whereas the *K_m* for beef kidney enzyme is unmeasurable because it shows no activity, even at concentrations of D-Ala approaching its solubility limit of 1.2 M (D'Aniello *et al.*, 1993b; Pollegioni *et al.*, 1993).

Low levels of DAAO activity can be found in mammalian tissues (ranging from 0.81 to 780 mU/g wet tissue (Weimar and Neims, 1977; D'Aniello *et al.*, 1993a), however endogenous DAAO is localized to the peroxisomal matrix where high catalase activity can detoxify the H₂O₂ by-product. In fact, all known DAAO sequences contain type 1 peroxisomal targeting sequences (Faotto *et al.*, 1995). This co-localization of endogenous DAAO and catalase, along with its relatively low specific activity, is likely to account for the minimal toxicity of D-amino acids in mammals. This is evidenced by the fact that administration of high doses (2 grams/kg body weight) of D-Ala, a substrate of choice for *R. gracilis* DAAO, produced no histopathological changes in rat kidney proximal tubule cells, where the highest level of DAAO activity is found (Kaltenbach *et al.*, 1979).

The practical application of ROS-generating enzyme systems has also been hampered by difficulty in enzyme delivery. Advances in gene transfer technology offer the possibility of overcoming this shortcoming by introducing the cDNAs encoding enzymes into malignant cells. Such cancer gene therapy paradigms depend on expressing genes encoding nonmammalian enzymes that convert pharmacologically inert prodrugs to highly toxic metabolites. *Rhodotorula gracilis* DAAO is well suited to this delivery approach because unlike GO and XO, its constitutive expression is not anticipated to be cytotoxic due to the lack of endogenous substrates. Furthermore, H₂O₂ is relatively stable, membrane permeable, and is cytotoxic to both proliferating and quiescent cells. These characteristics are potential advantages over the cytotoxic agents produced by a number of other enzyme/prodrug combinations that have been designed for gene-directed enzyme prodrug therapy (GDEPT) protocols (Connors, 1995).

In the present study, we investigated GDEPT using the *R. gracilis* DAAO cDNA. We have successfully expressed active *R. gracilis* DAAO lacking its carboxy-terminal peroxisomal targeting sequence in rat 9L gliosarcoma cells. Overexpression of cytoplasmically targeted DAAO in 9L tumor cells (Fig. 1, *Rxn. 1*) conferred toxicity to D-Ala exposure. We have attributed this cytotoxicity to the induction of oxidative stress by overproduction of H₂O₂ leading to generation of hydroxyl radical (Fig. 1, *Rxn. 2*) by demonstrating that DAAO-expressing cells respond to D-Ala exposure by stimulating glucose flux through the pentose phosphate pathway (PPP), which is linked to glutathione peroxidase removal of H₂O₂ (Fig. 1, *Rxns. 4–6*). Additionally, extracellular H₂O₂ was detected in the incubation buffer of DAAO-expressing cells following exposure to D-Ala. Finally, DAAO-expressing cells were further sensitized to D-Ala by depletion of reduced glutathione (Fig. 1, *Rxn. 7*), further implicating oxidative stress as the mechanism of cytotoxicity. These data demonstrate the concept of

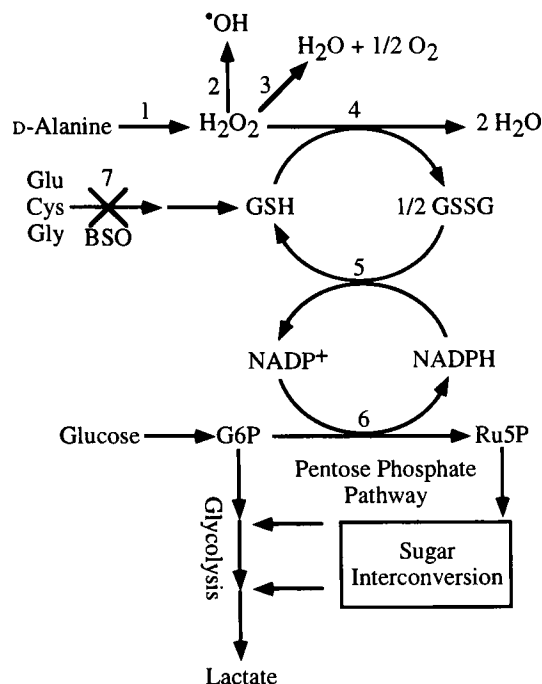


FIG. 1. Schematic representation of H_2O_2 metabolism in cells expressing DAAO. Intracellular H_2O_2 generated by DAAO (Rxn. 1) can be reduced to hydroxyl radicals via transition metal catalyzed Haber-Weiss chemistry (Rxn. 2). Hydroxyl radical reacts with DNA, lipids, and protein, inducing cell death. Alternatively, H_2O_2 can be removed by catalase (catalase, Rxn. 3) in the peroxisomes or by glutathione peroxidase (GPx, Rxn. 4) in the cytosol or at the plasma membrane. The GSH consumed in the second reaction is regenerated by glutathione reductase (Rxn. 5), using NADPH produced by the oxidative branch of the PPP as reducing equivalents (Rxn. 6). GPx activity can be inhibited by depleting the *de novo* synthesis of GSH with L-buthionine-(S,R)-sulfoximine (BSO), a specific inhibitor of γ -glutamylcysteine synthetase (Rxn. 7).

GDEPT with an ROS-generating oxidase for the treatment of neoplastic disease.

MATERIALS AND METHODS

Plasmid construction

The 1.2-kb *Eco* RI fragment from the plasmid pKK223.3-DAAO encoding the *R. gracilis* DAAO cDNA (GenBank U60066) (Pollegioni *et al.*, 1997) was ligated into the poly-cloning site of the selectable eukaryotic expression vector pcDNA3.1⁺ (Invitrogen) to construct pcDAO. A high-fidelity PCR mutagenesis strategy employing a mixture of *Pwo* and *Taq* polymerases (Expand, Boehringer Mannheim) was used to insert a eukaryotic translation initiation sequence (Kozak, 1989) and a unique *Cla* I site for screening. Two overlapping mutagenic oligonucleotides containing the Kozak sequence and *Cla* I site and two flanking oligonucleotides, one complementary to pcDNA3.1 and containing a unique *Nde* I site and the other complementary to a unique *Sac* II site in DAAO, were used to

amplify 0.5-kb and 0.6-kb products. Aliquots from these separate reactions were combined in a subsequent PCR amplification to produce a 1.1-kb product that was digested with *Nde* I and *Sac* II and ligated in the similarly digested template pcDAO. The resulting plasmids were screened for the mutation by digestion with *Cla* I. Mutagenesis was confirmed and PCR amplification misincorporations were checked by automated sequencing (Biomedical Research Core Facilities, UMMC) of the plasmid pcKDAO. The carboxy-terminal Ser-Lys-Thr type-1 peroxisomal targeting sequence (PTS) of the DAAO insert was removed by mutating the TCG codon encoding the Ser to an amber stop codon with the Altered Sites II *in vitro* Mutagenesis System (Promega). Codon substitution was confirmed by automated sequencing and the mutagenized insert was recloned into the *Eco* RI site of pcDNA3.1 to make pcKDAOc. All mutants were also subcloned into bacterial expression vectors with native 5' untranslated sequence.

Cell culture, stable transfection, and cellular lysates

Rat 9L gliosarcoma cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics at 37°C in a humidified incubator with a 5% CO_2 /95% air atmosphere. Cells were split by trypsinization once a week and were discarded after 30 passages. Exponentially growing cells were split 1:10 onto 10-cm tissue culture dishes the day before transfection with 30 μg of pcKDAOc using the standard calcium phosphate precipitation method (Ausbel *et al.*, 1994). Stable transfectants were selected by growth in 0.5 mg/ml G418 and individual clones were isolated on trypsin-impregnated filter paper disks (Domann and Martinez, 1995). Total cellular lysates were prepared by lifting adherent cells in 40 mM Tris, 1 mM EDTA, 150 mM NaCl pH 7.4 buffer. For Western blotting, cell pellets were resuspended in 250 mM Tris pH 7.6 and lysed by brief sonication on ice. For enzyme assays, cell pellets were resuspended in 50 mM $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ pH 8.3 and briefly sonicated. All extract supernatants were clarified by two sequential microcentrifugation steps at 4°C.

Immunoblotting

Equal amounts of protein, as determined by the bicinchoninic acid (BCA) protein assay (Pierce), were resolved by SDS/PAGE and transferred to nitrocellulose membranes (Laemmli, 1970; Towbin *et al.*, 1979). Immunodetection of DAAO was performed using affinity-purified rabbit polyclonal antibodies raised against purified *R. gracilis* DAAO holoenzyme (Perotti *et al.*, 1991). A peroxidase-conjugated goat-anti-rabbit immunoglobulin G (IgG) secondary antibody (Sigma) was used with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue-tetrazolium chloride to visualize the antigen-antibody complex.

Enzyme and glutathione assays

DAAO activity was measured with a horseradish peroxidase (HRP)-coupled, spectrophotometric assay using *o*-dianisidine as a reducing chromogenic HRP substrate (Bernt and Bergmeyer, 1974). Catalase (EC 1.11.1.6) activity was determined by following the decrease in H_2O_2 absorbance in 240

nm at 37°C (Aebi, 1984). The catalase reaction is a first-order reaction in both enzyme and substrate, precluding the definition of an international catalase unit. In this study, one catalase unit is defined as the activity that will decompose 1 μ mole of H₂O₂ per minute at pH 7.0 and 37°C when the H₂O₂ concentration falls from 10.3 to 9.2 mM. GPx (EC 1.11.1.9) activity was assayed by a glutathione reductase-coupled, spectrophotometric assay of NADPH absorbance (Flohe and Gunzler, 1984). Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity was measured spectrophotometrically as an increase in NADPH absorbance at 340 nm (Sigma Diagnostics Kit). Reduced glutathione levels were quantitated spectrophotometrically using the GSH-400 assay (Bioxytech, Marne, France). All assays were performed with Perkin-Elmer Lambda 6 or 14 spectrophotometers. Protein quantitation was performed with the BCA assay.

Cytotoxicity assay

An automated microculture assay employing the protein-binding dye sulforhodamine B (SRB, Sigma) was used to quantitate growth (Rubenstein *et al.*, 1990; Ben-Yoseph and Ross, 1994). Cells plated in 96-well culture plates at a density of 1,000–2,000 cells per well were allowed to grow for 24 hr, washed twice with Krebs–Ringer bicarbonate buffer (KRB), and exposed to increasing concentrations of H₂O₂ or D- or L-Ala in KRB. Following the exposure period of 1–24 hr, cells were gently washed twice and allowed to grow in MEM with FBS and antibiotics until untreated control wells reached subconfluence, generally after 4–7 days. The cells were then fixed and stained, and the absorbance at 540 nm was measured with an AutoReader microtiter plate reader (Cayman Chemicals). Toxicity was assessed as the fractional cell survival relative to unexposed controls. At least 16 replicates of each experimental condition were performed. For BSO sensitization studies, cells plated in 96-well plates were cultured overnight in MEM with serum, and subsequently incubated with 1 mM L-buthionine-(S,R)-sulfoximine (BSO) in MEM with serum for 24 hr prior to D-Ala exposure. IC₅₀ values were calculated by non-linear least-squares regression to a four-parameter logistic function using SigmaStat software (Jandel Scientific).

H₂O₂ assay

A modification of the phenol red oxidation assay (Pick and Keisari, 1980) was used to quantitate H₂O₂ release by cultured cells. Briefly, cells were grown to subconfluence in six-well plates, washed twice with KRB, and incubated with 1 ml of KRB containing phenol red, HRP, and increasing concentrations of D-Ala for 1 hr. The buffer was subsequently removed, alkalized with 1 N NaOH, and clarified by centrifugation, and the absorbance of oxidized phenol red was measured at 610 nm. Standard curves were generated by addition of 100 \times solutions of H₂O₂ in KRB to wells without D-Ala. H₂O₂ production was expressed as nmol/hr per mg cellular protein. Under these conditions the assay was linear over the 1–200 nmol/hr per mg range.

Pentose phosphate pathway flux

The method for determination of pentose phosphate pathway (PPP) activity has been previously described (Ben-Yoseph *et al.*, 1994b; Ross *et al.*, 1994). Briefly, metabolism of (1,6-

¹³C_{2,6,6-²H₂glucose (Omicron Biochemicals, South Bend, IN) by glycolysis or the PPP produces different ratios of lactate isotopomers that are extruded into the medium and quantitated by selected ion-monitored gas chromatography/mass spectrometry (GC/MS). Confluent cells in 24-well culture plates were washed twice and incubated for 1 hr in KRB with 5.5 mM labeled glucose and varying concentrations of H₂O₂ or D-Ala. Triplicate wells were used for each experimental condition and samples from each well were analyzed in triplicate. PPP values were corrected for total lactate produced when increasing concentrations of H₂O₂ or D-Ala inhibited lactate production. PPP stimulation was expressed as the percentage of glucose above basal flux that was metabolized through the PPP relative to glycolysis to lactate.}

RESULTS

Experiments with extracellular DAAO/D-Ala

Initial experiments were designed to examine the cytotoxicity of DAAO and D-Ala using purified enzyme. Exposure of 9L cells to increasing activities of purified *Trigonopsis variabilis* DAAO in the presence of enzyme-saturating concentrations of D-Ala revealed dose-dependent toxicity (IC₅₀ of 0.5 mU/well). Toxicity was completely abrogated by the addition of excess bovine catalase (Fig. 2), indicating that NH₃, the other product of the DAAO reaction, does not significantly contribute to the observed cytotoxicity.

Expression of *R. gracilis* DAAO in 9L cells

The next series of experiments were designed to evaluate the feasibility of expressing cytoplasmically targeted *R. gracilis* DAAO in mammalian cells. The *R. gracilis* DAAO cDNA was subcloned into the pcDNA3.1 expression vector under cytomegalovirus (CMV) promoter/enhancer and mutagenized to introduce a consensus eukaryotic translation initiation (Kozak)

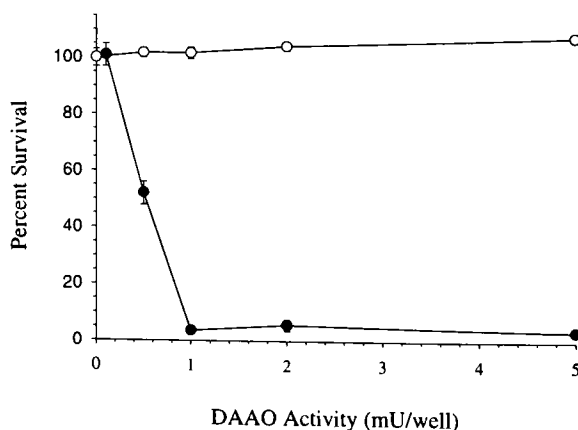


FIG. 2. Cytotoxicity of extracellular DAAO. Cultured 9L cells were exposed to increasing amounts of purified *Trigonopsis variabilis* DAAO in the presence of 20 mM D-Ala for 1 hr in the absence (●) or presence (○) of 4,000 units of purified bovine catalase. Values represent the means \pm SE ($n = 8$ wells).

sequence upstream of the start codon. Introduction of the consensus Kozak sequence resulted in mutation of His2 to Asp to place a G in the +4 position, which immediately follows the start codon (Kozak, 1989). A synonymous G-to-C point mutation in codon 102 encoding glycine was also produced during the polymerase chain reaction (PCR) mutagenesis. When expressed in *Escherichia coli*, the His2-Asp mutant had a specific activity in the crude extract similar to the wild-type recombinant enzyme. Site-directed mutagenesis was also used to remove the carboxy-terminal type-1 peroxisomal targeting sequence (PTS1). The PTS1-truncated DAAO mutant was expressed in *E. coli* and purified to homogeneity (Campaner *et al.*, 1998). This mutant DAAO is also fully active, characterized by a specific activity on D-Ala of 103 U/mg protein at 25°C, an apparent K_m of 0.7 mM (125 U/mg protein and 0.83 mM, respectively, for the wild-type DAAO). The mutant also dimerized normally and bound its FAD co-enzyme with an affinity typical of the wild-type enzyme.

Stably transfected 9L cells were screened qualitatively for cytotoxicity induced by exposure to 5 mM D-Ala for 24 hr. A clone showing significant sensitivity to D-Ala, 9Ldaao17, was chosen for further analysis. Immunoblotting of sonicated 9Ldaao17 cell extracts with a polyclonal anti-*R. gracilis* DAAO antibody showed the presence of an approximately 40-kD protein in the transfected cells (Fig. 3, inset lane 3) that co-migrates with purified recombinant *R. gracilis* DAAO produced in *E. coli* (lane 1). Also present is an approximately 45-kD endogenous protein in parental 9L cells (lane 2) that is recognized by the polyclonal antibody, which serves as a convenient gel-loading control. A peroxidase-coupled spectrophotometric assay showed that DAAO activity in crude 9Ldaao17 cell extracts was 8.8 ± 0.3 U/100 mg protein (mean \pm SE, $n = 5$), which corresponds to approximately 0.06% of the soluble proteins, assuming wild-type DAAO specific activity. No DAAO activity was observable in untransfected 9L cells. Moreover, using D-Ala as a substrate, the DAAO expressed in 9Ldaao17 was found to have an apparent K_m of 1.1 mM (Fig. 3). This value is in agreement with the measured K_m value for DAAO purified from *R. gracilis* cells and recombinant wild-type enzyme from *E. coli* (Pollegioni *et al.*, 1992; Campaner *et al.*, 1997).

Antioxidant enzyme activities

Spectrophotometric assays were employed to determine the activities of GPx and catalase, the two major H₂O₂-detoxifying enzymes in lysates of 9Ldaao17 cells (Fig. 1). GPx activity was estimated to be 0.6 ± 0.1 U/100 mg cellular protein (mean \pm SD, $n = 3$ separate cellular lysates). Catalase activity was estimated to be 150 ± 18 U/100 mg cellular protein (mean \pm SD, $n = 3$ separate cellular lysates) with a starting H₂O₂ concentration of 10.3 mM. Although G6PDH does not directly detoxify H₂O₂, it is linked to the GPx-mediated decomposition of H₂O₂ via glutathione reductase and is the rate-determining step in NADPH production by the PPP (Fig. 1). G6PDH activity in 9Ldaao17 extracts was determined to be 9.1 ± 2.1 U/100 mg protein (mean \pm SD, $n = 3$ separate cellular lysates).

Cytotoxicity studies

Displayed in Fig. 4A are the results from representative SRB cytotoxicity experiments. Exposure of 9Ldaao17 cells to in-

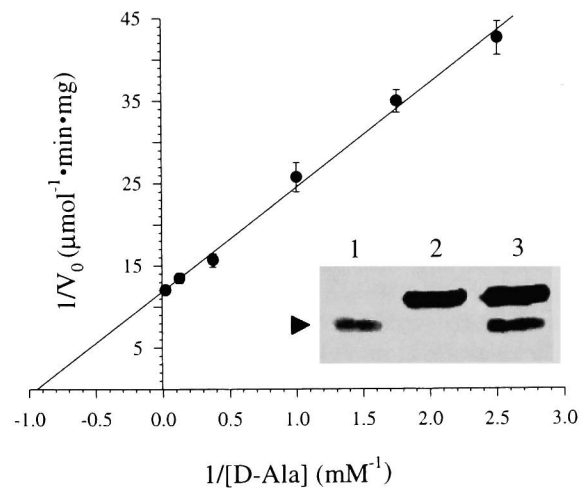


FIG. 3. Lineweaver-Burk plot of DAAO activity in crude lysates of 9Ldaao17 cells, where the x -axis intercept of the linear regression ($r^2 = 0.99$) equals $-1/K_m$. Cells were lysed as described in pyrophosphate buffer and DAAO activity was measured at increasing concentrations of D-Ala. (Inset) Immunoblot analysis with polyclonal anti-*R. gracilis* DAAO antibody. Purified *R. gracilis* DAAO (lane 1, 0.75 ng) was resolved by a 15% SDS/PAGE with 4.5 μ g of soluble protein from untransfected 9L cells (lane 2) or 9Ldaao17 cells (Lane 3). The *R. gracilis* DAAO band is indicated by the arrowhead.

creasing concentrations of D-Ala for 24 hr resulted in dose-dependent cytotoxicity with an IC₅₀ of 2.6 ± 0.7 mM (mean \pm SD, $n = 12$). Toxicity to parental 9L cells was minimal with significant cytotoxicity occurring only at hyperosmolar D-Ala concentrations (Fig. 4A). Growth of 9Ldaao17 was not inhibited by L-Ala (93% survival at 40 mM; data not shown). In addition, preincubation of 9Ldaao17 cells with 1 mM BSO for 24 hr caused a left shift in the dose-response curve (Fig. 4A), resulting in a new IC₅₀ of 0.4 ± 0.2 mM D-Ala (mean \pm SD, $n = 11$). Spectrophotometric assays revealed that BSO treatment depleted GSH levels in 9Ldaao17 cells by $90 \pm 17\%$ (mean \pm SD, $n = 3$). BSO did not enhance D-Ala cytotoxicity for 9L parental cells (data not shown). D-Alanine-induced cytotoxicity in 9Ldaao17 cells was also time-dependent (Fig. 4B).

Oxidative stress

The phenol red oxidation assay was used to measure production of extracellular H₂O₂ by cells cultured in the presence of D-Ala for 1 hr. 9Ldaao17 cells produced increasing amounts of H₂O₂ in response to increasing concentrations of D-Ala (Fig. 5A). Extracellular concentrations of H₂O₂ began to increase significantly at D-Ala concentrations between 5 and 10 mM. In contrast, parental 9L cells did not produce H₂O₂ in the presence of D-Ala.

Stimulation of PPP activity was used as a metabolic indicator of H₂O₂ detoxification (Ross *et al.*, 1994). When 9Ldaao17 cells were exposed to D-Ala for 1 hr, a dose-dependent stimulation of the PPP was observed with a maximal stimulation of $15.8 \pm 1.5\%$ above basal activity achieved between 10 and 20

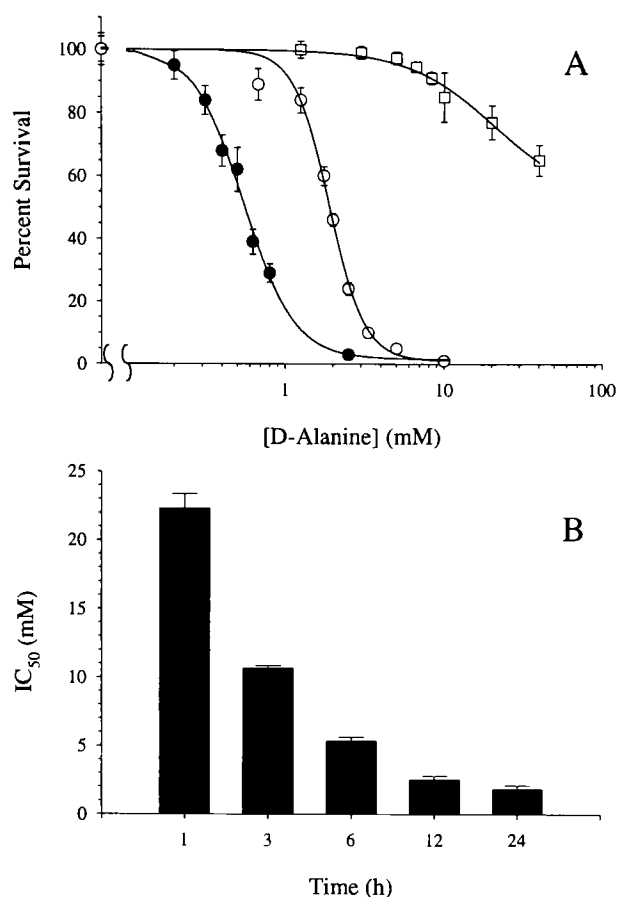


FIG. 4. Sensitivity of 9Ldaao17 cells to D-Ala. A. Untransfected 9L cells (□) and 9Ldaao17 cells with (●) or without (○) a 24-hr pretreatment with 1 mM BSO were exposed to increasing concentrations of D-Ala for 24 hr. Values represent the mean \pm SE ($n = 8$ wells) from representative experiments. B. Time dependence of D-Ala growth inhibition. 9Ldaao17 cells were incubated with increasing concentrations of D-Ala for 1, 3, 6, 12, or 24 hr. The IC₅₀ values (\pm SE, $n = 8$ wells per concentration) are from single representative experiments.

mM D-Ala (Fig. 5B). In contrast, D-Ala at concentrations as high as 40 mM did not elicit significant stimulation of glucose flux through the PPP in parental 9L cells (Fig. 5B; maximal stimulation $1.6 \pm 0.5\%$). Moreover, expression of *R. gracilis* DAAO in 9Ldaao17 cells did not significantly affect the basal PPP activity (data not shown). Bolus addition of H₂O₂ also stimulated PPP glucose flux in 9Ldaao17 cells in a dose-dependent fashion, reaching a saturation level of $19.3 \pm 1.5\%$ above baseline at about 600 μ M (Fig. 5C), which is consistent with the maximal H₂O₂-induced stimulation of the PPP in parental 9L cells (Ben-Yoseph *et al.*, 1994a). It should also be noted that extracellular addition of purified DAAO stimulated PPP activity in the presence of D-Ala to the same extent as preformed H₂O₂ (data not shown). Furthermore, this stimulation was completely blocked by inclusion of 4,000 units of catalase in the incubation buffer.

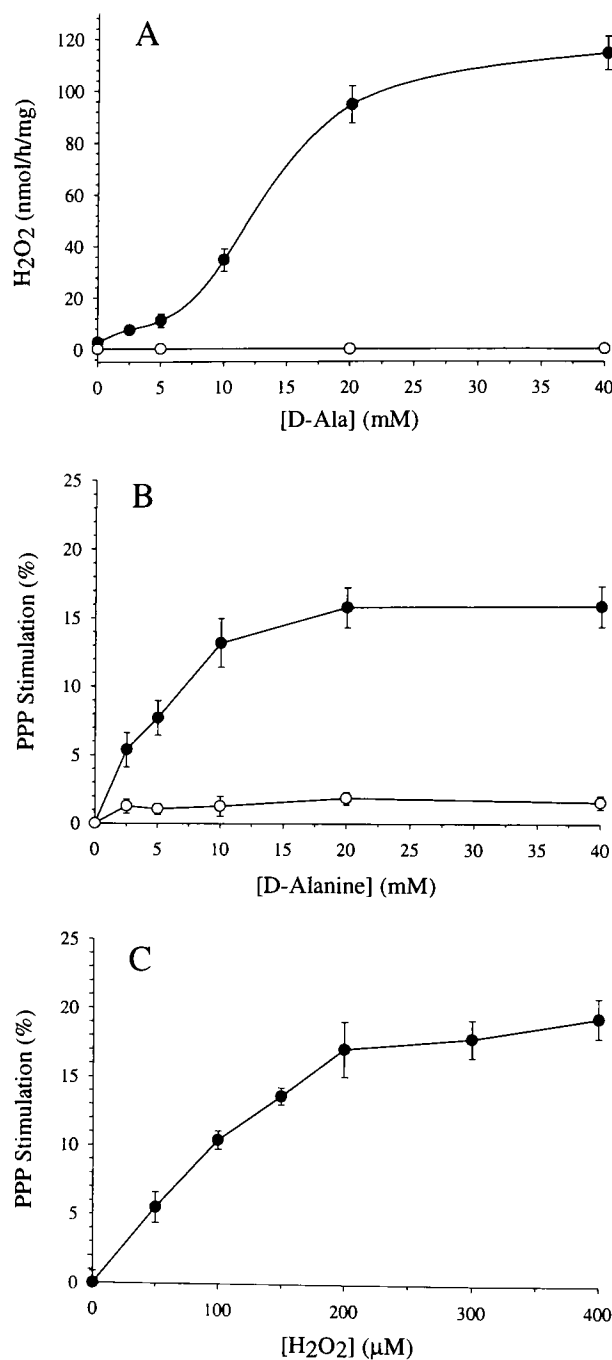


FIG. 5. H₂O₂ production and metabolism by 9Ldaao17 cells. A. Extracellular H₂O₂ production by parental 9L cells (○) and 9Ldaao17 cells (●) cultured in six-well plates with increasing concentrations of D-Ala. Values are means \pm SE from a representative experiment ($n = 6$ wells per concentration). B. The glucose flux through the PPP was monitored by GC/MS analysis of lactate isotopomers extruded from 9L cells (○) and 9Ldaao17 cells (●) cultured in 24-well plates with (1,6-¹³C₂,6,6-²H₂)glucose and increasing concentrations of D-Ala for 1 hr. C. Stimulation of glucose flux through the PPP by bolus addition of increasing concentrations of preformed H₂O₂. Values for all PPP assays are means \pm SE ($n = 9$ wells per concentration from three separate experiments).

DISCUSSION

A major problem in the clinical management of cancer patients is the limited differential toxicity of chemotherapeutic agents and radiation toward neoplastic versus normal cells. Gene transfer technology offers the possibility of surmounting this limitation by introducing unique drug targets into malignant cells. GDEPT paradigms for cancer gene therapy depend on expressing genes encoding nonmammalian enzymes that convert pharmacologically inert prodrugs to highly toxic metabolites. The studies reported here demonstrate the potential of intracellular expression of *R. gracilis* DAAO together with administration of D-Ala as a GDEPT protocol.

Prior to conducting transfection experiments, the cytotoxicity of purified DAAO against 9L cells was tested. In the presence of D-Ala, extracellular DAAO was cytotoxic at low levels of activity and with brief periods of exposure (Fig. 2). The IC_{50} was 0.5 mU/well, which over the 1-hr incubation period is calculated to produce 0.03 μ mol of H_2O_2 , corresponding to a cumulative concentration of 150 μ M. This is consistent with the 60 μ M IC_{50} for preformed H_2O_2 previously reported for 9L cells (Ben-Yoseph and Ross, 1994) allowing for the difference between the bolus addition of preformed H_2O_2 versus its enzymatic production by extracellular DAAO over 1 hr.

To produce cytoplasmic DAAO, the cDNA encoding *R. gracilis* DAAO was truncated to remove its type-1 peroxisomal targeting sequence. The altered cDNA directed production of functional DAAO in both *E. coli* and 9L $daao17$ cells, indicating that its peroxisomal targeting sequence is not required for enzymatic activity (Campaner *et al.*, 1998). Similarly, expression of the mutant enzyme in 9L $daao17$ cells did not significantly alter its apparent K_m for D-Ala using the crude extract (Fig. 3). We attempted to confirm the cytoplasmic localization of the mutant DAAO in 9L $daao17$ cells with immunofluorescence microscopy. DAAO appeared to be located in the cytoplasm (data not shown), however, definitive assignment of a cytoplasmic localization will require epitope-tagging of *R. gracilis* DAAO because 9L cells contain an unidentified protein that cross-reacts with the affinity-purified rabbit antisera to the yeast homolog (Fig. 3, inset). The observation that cytotoxicity occurs in 9L $daao17$ cells despite the presence of 150 U/100 mg of catalase further implies cytosolic localization of DAAO. This is consistent with the ability of 8.8 U/100 mg of DAAO to overwhelm the detoxification capacity of 0.6 U/100 mg of GPx in the cytoplasm of 9L $daao17$ cells. The fact that the maximal stimulation of glucose flux through the PPP by D-Ala and preformed H_2O_2 in 9L $daao17$ cells was similar (Fig. 5B,C) further supports this interpretation. Additionally, extrusion of H_2O_2 from 9L $daao17$ cells dramatically increased at concentrations of D-Ala between 5 and 10 mM (Fig. 5A). This correlates with the concentration of D-Ala at which PPP stimulation was maximal (Fig. 5B), indicating that GPx-mediated H_2O_2 removal was saturated. Thus, the intracellular half-life of H_2O_2 would be expected to be prolonged, providing a greater probability for its diffusion out of the cell. The presence of extracellular H_2O_2 suggests that H_2O_2 produced by transduced cell "factories" should kill adjacent untransduced cells in a process known as "bystander" killing (Freeman *et al.*, 1993).

The toxic product of this therapeutic strategy, H_2O_2 , is toxic

to both proliferating and quiescent cells (Vidair and Dewey, 1991); in contrast, the most commonly investigated GDEPT protocols generate cytotoxic agents that are specific for actively proliferating cells (Connors, 1995). In support of the toxicity of H_2O_2 for nonproliferating cells, we have conducted qualitative experiments in which confluent 9L $daao17$ cells were exposed to 10 mM D-Ala overnight. Under these conditions, the cells were killed and detached from the culture plate (data not shown). The ability to kill noncycling cells is an attractive feature because a substantial fraction of cells within solid tumors may be quiescent (Yoshii *et al.*, 1986). Although H_2O_2 has been shown to induce programmed cell death in a number of cell types, apoptosis may not be the mechanism underlying the cytotoxicity of DAAO gene therapy due to the relatively large amounts of intracellular peroxide generated. Further investigations are currently underway to elucidate the mechanism of cell death.

In the context of primary CNS malignancies, we have previously shown that cultured glioma cells are intrinsically more susceptible to oxidative injury induced by H_2O_2 ($IC_{50} = 60 \mu$ M) than cultured primary neuronal ($IC_{50} = 100 \mu$ M) or astrocytic cells ($IC_{50} = 600 \mu$ M) (Ben-Yoseph and Ross, 1994). A recent study also described an inverse correlation between the histological grade and antioxidant enzyme activities in a series of human central nervous system (CNS) malignancies (Pu *et al.*, 1996). The authors of this study also reported that the mean GPx activity in 17 high-grade human gliomas was 1.1 ± 0.2 U/100 mg, which is comparable to the levels we observed in 9L cells. Additionally, a wide array of agents that interfere with cellular antioxidant repair mechanisms are available. These include enzyme inhibitors, thiol-depleting drugs, and DNA-repair inhibitors, all of which have been used as radiosensitizers. We previously reported that preincubation of 9L cells with BSO resulted in a three-fold sensitization to H_2O_2 -induced toxicity ($IC_{50} = 20 \mu$ M), thereby increasing their differential toxicity to H_2O_2 to five- and thirty-fold more than primary neuronal and astrocytic cultures, respectively (Ben-Yoseph and Ross, 1994; Ben-Yoseph *et al.*, 1994a). Similarly, the studies described herein showed that BSO further sensitizes 9L $daao17$ cells to D-Ala (Fig. 4A). The ability to sensitize cells further to the DAAO gene therapy protocol with a wide variety of agents is unique among GDEPT strategies and is particularly exciting for application to neuro-oncology because administration of BSO to mice bearing intracranial gliomas resulted in a selective depletion of GSH in the tumors while normal brain GSH levels remained intact (Skapek *et al.*, 1988). This is especially promising because cellular glutathione content rather than antioxidant enzyme activities was the main predictor of the H_2O_2 sensitivity of six murine tumor models (Nathan *et al.*, 1980).

The application of this negative selective system to *in vivo* cancer gene therapy awaits the results of a number of experiments. Most importantly, the pharmacokinetic properties of D-amino acids must be characterized to devise appropriate dosing schedules for evaluating the efficacy of the DAAO gene therapy approach in animal models. Eventually we hope that DAAO gene therapy will prove synergistic with multimodality brain tumor treatment protocols including surgery, radiation, and chemotherapy. The fact that carmustine (BCNU), the mainline chemotherapeutic for malignant gliomas, is a potent and

specific inhibitor of glutathione reductase and has been shown to sensitize tumor cells to H₂O₂ up to 18.7-fold (Nathan *et al.*, 1980) supports this assertion.

In conclusion, this study demonstrated that the cytotoxicity of ROS can be harnessed by the expression of DAAO in tumor cells by gene transfer techniques. Exposure of tumor cells to D-Ala resulted in the cytotoxic overproduction of ROS. This novel gene therapy paradigm possesses several potential advantages over other GDEPT systems. (i) The H₂O₂ product is cell cycle nonspecific. (ii) H₂O₂ has similar cell permeability characteristics to water, thus providing the rationale for achieving bystander killing. (iii) Tumor cells can be presensitized to oxidative damage prior to treatment with DAAO/D-Ala. (iv) The D-amino acid substrates for DAAO (*e.g.*, D-Ala) are essentially nontoxic.

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