

# Transfection of the DAAO gene and subsequent induction of cytotoxic oxidative stress by D-alanine in 9L cells

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**Abstract.** D-amino acid oxidase (DAAO) can catalyze the dehydrogenation of D-amino acids, such as D-alanine, to the corresponding amino acids and is then reoxidized by molecular oxygen to yield hydrogen peroxide, a reactive oxygen species, which reacts with DNA, lipids and protein, inducing cell death. This study investigated whether rat glioma 9L cells infected with the recombinant retrovirus containing the DAAO cDNA fragment can be induced in order to undergo cytotoxic oxidative stress by D-alanine. The recombinant retroviral vector, plzrus-DAAO-FLAG-GFP (pl-Dfg), was constructed and used to transfect packaged phoenix cells. The supernatant containing recombinant retroviral particles from the transfected phoenix cells was harvested and utilized to infect target 9L cells. The cytotoxic oxidative stress of infected 9L cells was induced by the DAAO substrate, D-alanine. The plasmid pl-Dfg was successfully constructed. The high titer retroviral supernatant was obtained from the transfected phoenix cells. Infected 9L cells were less viable after exposure to D-alanine compared to the control group. Anti-apoptotic proteins significantly inhibited cell death. The DAAO/D-alanine system has a potential utility for gene therapy and may be an effective strategy for the treatment of brain cancer and other malignant tumors.

## Introduction

Gene-directed enzyme prodrug therapy (GDEPT) or virus-directed enzyme prodrug therapy (VDEPT) is an antineoplastic treatment strategy, which uses selectively replicating viruses as vectors to introduce target suicide genes encoding enzymes

specific to tumor cells, which are capable of converting prodrugs to cytotoxic drugs (1,2). D-amino acid oxidase (DAAO) is a flavoprotein containing a coenzyme a non-covalently bound molecule of flavin adenine dinucleotide (FAD) per 40 kDa protein monomer. It catalyzes the dehydrogenation of D-amino acids, such as D-alanine, to the corresponding amino acids, which spontaneously hydrolyze to  $\alpha$ -keto acids and ammonia. The reduced FAD cofactor is then reoxidized by molecular oxygen to yield hydrogen peroxide ( $H_2O_2$ ), which belongs to the reactive oxygen species (ROS) (3-5). The oxidative agent has a deleterious effect on cells, for example, it damages the cellular membrane by oxidizing lipid and breaks DNA strands (3-5). This GDEPT approach using DAAO, a suicide gene, and D-alanine as a substrate could be potentially useful for cancer gene therapy.

DAAO has been identified in various mammalian tissues at a low level and is not retained in many tumor cells (6), which suggests that the  $H_2O_2$ -producing enzyme, DAAO, together with substrate D-alanine is possibly useful for causing cell oxidative stress. In this study, we used DAAO/D-alanine as an enzyme/prodrug system to produce an oxidative agent in cancer cells, which causes cell oxidative stress. DAAO was first introduced into rat glioma 9L cells by a recombinant retrovirus. The infected 9L cells were then exposed to D-alanine in order to cause cell stress and death (Fig. 1). The aim of this study was to determine the potential utility of the DAAO/D-alanine system in cancer gene therapy.

## Materials and methods

**Construction of plasmid.** The 1.2 kb fragment was amplified from pcDNA3.1 DAAO (5) encoding the red yeast *Rhodotorula Gracilis* DAAO cDNA (GenBank U60066) by routine PCR. Two flanking specific primers were designed and synthesized. The forward primer was complementary to the 5' end of DAAO cDNA and the *MunI* restriction site was added. Another was complementary to the 3' end and the epitope tag FLAG replaced the stop codon with the *EcoRI* site added. The amplified PCR product *MunI*-DAAO-FLAG-*EcoRI* fragments were purified by using agarose gel directly inserted into a pre-cut plasmid plzrus-cd on *MunI* and *EcoRI* sites. The ligation mixtures were used to transform competent TM cells (Gibco, 10268-019). After functional testing, the plasmid with an insert was digested with *EcoRI*, purified on an agarose gel and ligated with a green fluorescent protein

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**Abbreviations:** DAAO, D-amino acid oxidase; GFP, green fluorescent protein; ROS, reactive oxygen species;  $H_2O_2$ , hydrogen peroxide

**Key words:** gene therapy, tumor, D-amino acid oxidase, D-alanine

(GFP) cDNA fragment from plasmid pTETRET-GFP digested with *EcoRI*. The insert was checked by the digestion of the plasmid with *EcoRI*. The correct orientation of the inserts was confirmed by PCR. The positive clone of plzrus-DAAO-FLAG-GFP (pl-Dfg) with correct orientation was selected for a large-scale preparation. The plzrusLacZ (pl-laz) served as a negative control, which had the same backbone vector as pl-Dfg.

**Preparation of recombinant retroviruses.** Packaged phoenix cells were used as virus-producing cells and maintained in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal calf serum at 37°C in a 5% CO<sub>2</sub> incubator. The cells were grown to 80% confluence and were transfected with 5 µg of the plasmid pl-Dfg using calcium phosphate-DNA coprecipitation with chloroquine or 30 µl of Lipofectamine Plus Transfection Reagent (Life Technologies, 10964-013), as described by the manufacturer. As a negative control, pl-laz was used to transfect the same cells under the same conditions. At 48 h post transfection, the supernatant containing recombinant retroviral particles was collected, centrifuged, filtered with 0.45-µm filters and frozen at -80°C or used immediately.

**Infection of 9L and other cell lines.** Target rat glioma 9L cells (5) were infected with the above supernatant. In order to estimate the specific effect of DAAO, we compared various other cell lines, including the human breast cancer cells, MCF7 and squamous cell carcinoma SCC. 9L and SCC cells were maintained in DMEM, while MCF7 cells were maintained in Roswell Park Memorial Institute (RPMI) medium. Both media contained 10% fetal calf serum. The day before infection, cells were placed in a 6-well plate with 50% confluence. When infection occurred, cells were incubated with 4 ml of infectious medium (50% retroviral supernatant, 50% fresh growth medium and 6 µg/ml polybrene <Sequabrene, Sigma s2667>). At 24 h post infection, the infectious medium was changed to a fresh culture medium. Cells were ready to assay for events of experimentation.

**DAAO assay.** The efficiency of 9L cells infected with viral supernatant was determined by the measurement of DAAO activity (5). The reaction of velocity is determined in a peroxidase-coupled system by continuous spectrophotometric recording of the increase in o-dianiside absorbance at 436 nm. Cells were lifted from a culture plate with a lift buffer, centrifuged and re-suspended in 0.1 ml reagent A (20 mM Na<sub>2</sub>H<sub>2</sub>P<sub>2</sub>O<sub>7</sub> Anhydrous <Sigma P-8135> pH 8.3). The cells were sonicated briefly on ice and the supernatant was then collected as cellular extracts. Reagent B (0.1 ml) (10 mg/ml HRP <Sigma P8250> in reagent A) and 2.8 ml reagent C (50 mM D-alanine, 0.0065% o-dianisidine <Sigma D3252> in reagent A) were equilibrated to 37°C. After 5 min, 0.1 ml of the cellular extracts was added and the optical density at 436 nm was determined. The activity of DAAO was calculated from the linear portion of the standard curves.

**Western blot analysis.** Equal amounts of cell lysates were resolved by SDS/PAGE and transferred to nitrocellulose

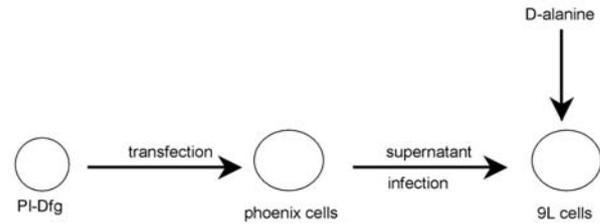


Figure 1. Experimental schedule. The recombinant retroviral vector, pl-Dfg, was constructed, then transfected to packaged phoenix cells, from which the supernatant was obtained and used to infect rat glioma 9L cells. The infected 9L cells were induced to undergo cytotoxic oxidative stress by the exposure to D-alanine.

membranes. Immunodetection of DAAO was performed using an anti-FLAG M2 monoclonal antibody (Sigma, F3165). A peroxidase-conjugated goat secondary antibody (Sigma, A 4416) was used with enhanced chemiluminescence 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue-tetrazolium chloride (ECL) to visualize the antigen-antibody complex.

**Senescence-associated X-gal stain.** When the vector, pl-Dfg, was transfected, pl-laz served as a negative control. In order to examine the reporter enzyme LacZ expression, X-gal staining was performed. At 48 h post transfection and infection, cells were rinsed with PBS, fixed in 0.1% glutaraldehyde for 5 min and washed with 1 mM MgCl in a PBS solution. X-gal solution was then added and was incubated with cells for 1 h. Stained blue cells were counted as positive.

**Cytotoxicity assay.** To induce cytotoxic stress, the infected 9L and parental 9L cells were exposed and not exposed to D-alanine at different concentrations. An automated microculture assay employing the protein-binding dye, sulforhodamine B (SRB) (Sigma), was used to quantitate the ratio of living to dead cells. For the assay, the cells were plated in a 96-well culture plate at a density of 2,000 cells per well and were allowed to grow for 24 h. After two washes with Krebs-Ringer bicarbonate buffer, the cells were exposed to increasing concentrations of D-alanine from 0 to 10 mM for 24 h. The cells were then fixed with trichloroacetic acid electrophoresis reagent, stained with SRB and solubilized with a pH 4.0 Tris buffer. The resulting optical density was determined at 540 nm for quantitation and at 690 nm for background correction in the AutoReader microtiter plate reader (Cayman Chemicals). Toxicity was assessed as the fractional cell survival relative to the unexposed control. After the above D-alanine dosage study, the concentration of 2.5 mM D-alanine was chosen to examine the cytotoxicity in MCF7 and SCC cells. The killing effects of D-alanine on these cells were determined as an automated microculture assay. Values represent the mean ± SE from two to three separate experiments (n=6-8 wells each).

**Establishment of stable infectants and induction of cytotoxic stress.** At 48 h after infection, 9L cells were grown in a selective medium (DMEM containing 5 µg/ml puromycin and 10% fetal calf serum) for two weeks. The transfectants were observed under a fluorescent microscope and the brightest

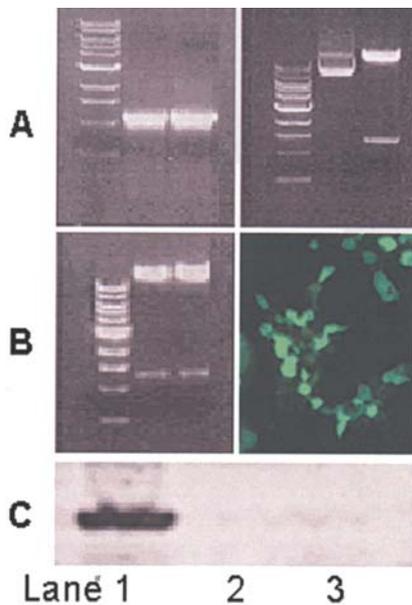


Figure 2. Construction of the recombinant retroviral vector pI-Dfg and preparation of a recombinant retrovirus containing DAAO cDNA. DNAs run on agarose gel. Each lane 1 of the 3 agarose gels was a DNA ladder. (A) Left: lanes 2 and 3, purified *Mum1*-DAAO-FLAG-*EcoRI* PCR product, 1.2 kb; Right: lanes 2 and 3, plzrus-cd without and with digestion; the size of vector backbone plzrus was 11.5 kb. (B) Left: lanes 2 and 3, digested pI-Dfg. The size of the GFP fragment was 1.5 kb. Right: Phoenix cells transfected with pI-Dfg were under the view of a fluorescent microscope. The green cells were positive transfectants. (C) Western blot. Lanes 1, 2 and 3: phoenix cells were transfected with pI-Dfg, pI-lacZ and none separately. Lane 1 shows expression of FLAG fusion protein with the molecular weight of 67 kDa.

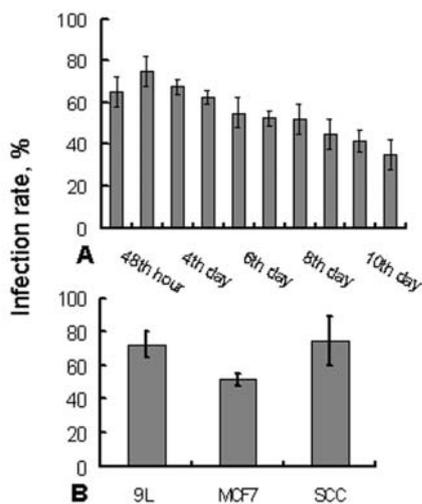


Figure 3. Infection rate. (A) Retroviral supernatant from phoenix cells at different dates of post-transfection was used to infect 9L cells. The virus produced from the 3rd day gave the highest infection rate. (B) The pooled viral-supernatant was infected to 9L, MCF7 and SCC cells. The infection rates between each cell line were not significantly different.

ones were chosen for continued growth and further analysis. To examine the properties of the cell line, DAAO activity, Western blot and observation of GFP expression under a fluroscope were performed. To induce cytotoxic stress, 2.5 mM of D-alanine was added to the cells for 24 h. Then the dead

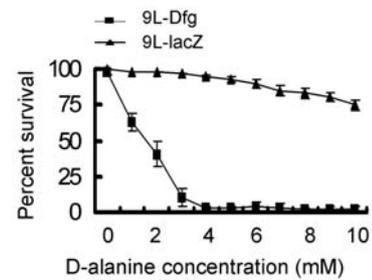


Figure 4. D-alanine dose-dependent cell killing. 9L cells infected with the target virus encoding the Dfg gene (9L-Dfg) and control virus encoding lacZ gene (9L-lacZ) were exposed to D-alanine at different concentrations. The dead cells were measured under an automated microculture assay.

and surviving cells were calculated according to an automated microculture assay.

**Apoptosis assay.** To prove the cell death was to commit suicide, an apoptosis assay was performed. MCF7 wild-type cells and MCF7 cells co-expressing anti-apoptotic proteins, including p35, bclxL and cramaA called MCFp35, MCF7, bclxL and MCFcraMA separately, were used to infect testing and control supernatant. They were then exposed or not to 2.5 mM D-alanine. The procedures of infection and exposure were the same as above. The dead cells were counted by an automated microculture assay.

## Results

**Construction of recombinant retroviral vector.** A plasmid pI-Dfg containing DAAO cDNA was successfully constructed. Correct DNA bands were run on an agarose gel (Fig. 2). The functions of pI-Dfg were examined (Fig. 2) by DAAO activity, immunoblotting and the observation of green cells under a fluorescent microscope on both transfected phoenix cells and infected 9L cells, demonstrating that they had an expression of the target DAAO gene and simultaneous expression of FLAG and GFP fusion proteins.

**Preparation of high titer retroviral supernatant.** The viral supernatant was obtained from transfected cells and was collected every day for 2 weeks starting 48 h post transfection. The cells then became unhealthy and detached from the dishes. The titer of the virus encoding the Dfg gene was determined by the evaluation of infected cells, which appeared fluorescent green. It was highest on day 3 post transfection and became lower gradually (Fig. 3). The sensitivities of 9L, MCF7 and SCC cells to the virus were slightly varied, though the infection rates did not reach a significant difference (Fig. 3). Meanwhile the titer of the virus encoding the negative control gene, LacZ, was obtained by the observation of blue cells after X-gal staining.

**D-alanine dose-dependent cell killing.** The exposure of D-alanine to infected 9L cells expressing DAAO markedly affected the viability of cells. Exposure to different concentrations of D-alanine (0 to 10 mM) of 9L cells infected with testing the supernatant for 24 h produced a dose-dependent killing of cells. The cell survival curve declined

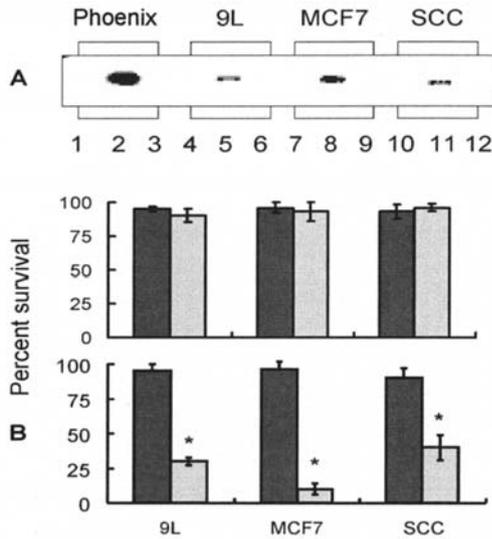


Figure 5. The effect of D-alanine on 9L, MCF7 and SCC cells expressing DAAO. (A) 9L, MCF7 and SCC cells expressing the DAAO fusion protein were examined with immunoblotting using an anti-FLAG antibody: Lanes 1-3, phoenix cells; lanes 4-6, 9L cells; lanes 7-9, MCF7 cells; lanes 10-12, SCC cells; lanes 1, 4, 7 and 10, wild-type cells; lane 2, transfected with pl-Dfg; lane 3, transfected with pl-lacZ; lanes 5, 8 and 11, infected with virus-Dfg; lanes 6, 9, and 12, infected with virus-lacZ. Lanes 2, 5, 8, and 11 had expression of FLAG fusion protein. (B) Effect of D-alanine on infected 9L, MCF7 and SCC cells. Top panel: 9L, MCF7 and SCC cells infected with the control supernatant were without (black bars) and with (gray bars) exposure to D-alanine. Bottom panel: 9L, MCF7 and SCC cells infected with target testing supernatant were without (black bars) and with (gray bars) exposure to D-alanine. The percentage of cell survival was measured. \*P<0.05.

linearly from 0 to 4 mM (Fig. 4). The dead cells shrank, floated and lost their green shade under a fluorescent microscope. However, in spite of exposure to increasing concentrations of D-alanine, wild-type 9L cells infected with the control supernatant were healthy and had experienced growth (Fig. 4).

**Induction of cytotoxic oxidative stress by D-alanine on infected 9L, MCF7 and SCC cells.** To confirm the specific effect of DAAO, other infectants including the MCF7 and SCC cell lines were used for comparison. According to the above D-alanine dosage study, a concentration of 2.5 mM was chosen to observe the cytotoxic reaction. With an exposure of 2.5 mM of D-alanine, the survival of cells infected with the testing supernatant was significantly lower compared to without exposure (Fig. 5). This cytotoxic stress occurred in both MCF and SCC cells. Whereas, in spite of exposure, the survival of parental cells and cells infected with control supernatant were not different (Fig. 5).

**Establishment of 9L stable infectants and induction of cytotoxic oxidative stress.** The DAAO gene was introduced into glioma 9L cells permanently. Infected 9L cells were grown in a selected medium to obtain stable cells expressing a DAAO fusion protein. The protein expression levels of stable cells were examined by DAAO activity, Western blotting with anti-FLAG antibody and green fluorescent cells showing that all three of them were positive. For DAAO activity (Fig. 6), absorbances of cell extracts had an initial up

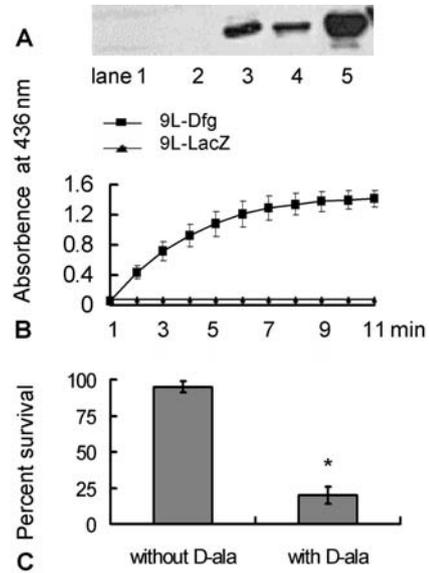


Figure 6. Establishment of 9L cells stable expressing DAAO and the effect of D-alanine. (A) Immunoblot: after the drug selection of the infected cells, four clones were examined. Three of them (lane 3-5) were positive; lane 1, parental 9L cells; lane 2, negative clone. (B) DAAO activity: Stable 9L-Dfg and negative control 9L-lacZ cells were measured for DAAO activity; 9L-Dfg had initial up linear portion, 9L-lacZ had none. (C) The percentage survival of stable 9L-Dfg cells was observed after cells that were and were not exposed to 2.5 mM of D-alanine. \*P<0.05.

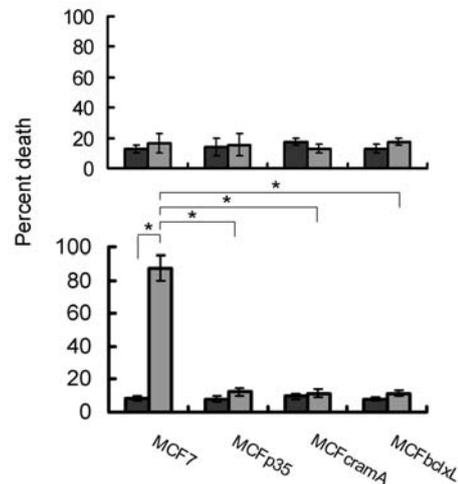


Figure 7. Apoptosis assay: MCF7 parental cells and MCF7 cells co-expressing anti-apoptotic proteins were infected with the control supernatant (top panel) and testing supernatant (bottom panel) that were without (back bars) and with (gray bars) exposure to D-alanine. The dead cells were then measured. Anti-apoptotic proteins significantly inhibited cell death induced by D-alanine in MCF cells expressing DAAO. \*P<0.05.

linear portion, the rate was 0.015-0.05 A<sub>436</sub>/min and the resulting color of the reaction was brown, demonstrating that 9L-Dfg stable cells did have DAAO activities; while absorbances of extracts from negative control 9L-LacZ cells were recorded as 0.000 or minus (e.g.-0.005), the curve was horizontal and the resulting reaction solution was colorless. For immunoblot, four clones were examined and three of them were positive (Fig. 6). To induce cytotoxic stress, the

 SPANDIDOS PUBLICATIONS cells were exposed to 2.5 mM of D-alanine for 24 h, with transient infection, the cells were significantly killed according to an automated microculture measurement (Fig. 6).

*Induction of apoptosis by DAAO and D-alanine.* To prove the cell death was to commit suicide, apoptosis assay was performed. The observation demonstrated that the anti-apoptotic proteins significantly inhibited cell death induced by D-alanine in MCF cells expressing DAAO (Fig. 7). The cultured cells showed signs of apoptosis such as cell shrinkage, development of bubble-like blebs on their surface, ballooning formation and breaking into small fragments under high magnification.

## Discussion

Malignant gliomas are the most common primary brain tumors accounting for 30-40% of all primary brain tumors (7,8). They are among the gravest forms of cancer and the prognosis is always poor. The average survival rate is between 12 and 18 months, with 90-95% of patients surviving <2 years, without the possibility of spontaneous remission or effective treatment (9,10). Compared to the incurability with conventional treatments of surgery, chemotherapy and radiotherapy, gene therapy for gliomas has proven to be successful. For malignant brain tumors, cytotoxic genes include the herpes simplex virus thymidine kinase (HSV-TK) (11,12) and cytosine deaminase (CD) (13). For HSV $tk$ /GCV therapy, the HSV- $tk$  gene is transduced into tumor cells and GCV is administered systematically. GCV is harmless to normal cells without HSV- $tk$ . When tumor cells express HSV- $tk$ , this enzyme converts GCV into a cytotoxic molecule, resulting in cell death (13). The procedure of this suicide gene therapy is very similar to the one described in our study of the DAAO/D-ala system. The HSV-TK vector system was limited by a low viral titer and low target cell transduction frequency; bystander cell killing needs to be improved and the overall results were disappointing (14,15). Kaliberov *et al* recently reported that combined treatment using adenoviral-directed enzyme/prodrug therapy bacterial CD gene (AdbCD-D314A)/5-fluorocytosine (5-FC) and radiation therapy has the potential to become a powerful method of cancer therapy (16). Further research for efficient treatment of brain tumors is needed.

In this study, DAAO was first introduced into glioma 9L cells by a recombinant retrovirus. The infected 9L cells were then exposed to D-alanine causing cell death. DAAO expression is harmless to 9L cells, but after exposure to cells of D-alanine for 24 h, the cells were mostly dead. Furthermore, this cytotoxic stress exerted by D-alanine was dose-dependent. In spite of exposure to D-alanine, 9L cells of the negative control were healthy and had experienced growth. To determine the specific effect of DAAO, we compared some other cell lines, including MCF7 and SCC cells. The killing effect of DAAO and D-alanine on these cells was consistent. This reaction is based on the oxidative stress of ROS, H<sub>2</sub>O<sub>2</sub>, which arises from the induction by D-alanine in cells expressing the DAAO protein. DAAO catalyzes the stereoselective oxidative deamination of D-amino acids via the following reactions (5): D-amino acid +

$H_2O + O_2 \rightarrow \alpha\text{-keto acid} + NH_3 + H_2O_2$ . H<sub>2</sub>O<sub>2</sub> is a ROS, which readily crosses cellular membranes and the hydroxyl radical reacts with DNA, lipids and protein, inducing cell damage and death. H<sub>2</sub>O<sub>2</sub> is relatively stable and membrane permeability allows it to diffuse a short distance, so that it is cytotoxic to both proliferating and quiescent cells. This bystander cell killing is beneficial to tumor treatment. In addition, retroviral gene transfer can produce a long-lasting therapeutic effect with a single treatment. Furthermore, the retroviral gene transduces preferentially dividing cells (17), which is beneficial in treating dividing glioma tumor cells, but not normal glial cells.

DAAO/Dalanine may kill cancer cells by the induction of apoptosis. In order to address this issue, an apoptosis assay was performed. The observations demonstrated that the anti-apoptotic proteins significantly inhibited cell death and the cultured cells showed signs of apoptosis of morphological changes. This proved that cells were killed by DAAO and D-alanine was associated with apoptosis, which lead cancer cells to commit suicide and proved that the utility for tumor-specific induction of apoptosis could be a powerful approach to cancer therapy. We did not evaluate the ROS status here, because from theories to experiments, many researchers have clearly shown that DAAO catalyzes the stereoselective oxidative deamination of D-amino acids via specific reactions and H<sub>2</sub>O<sub>2</sub> was produced by the DAAO/D-alanine system. Importantly, in this study, we used all necessary controls.

GFP is a revolutionary biomarker for monitoring gene expression and protein localization in real-time. In our case, this makes it possible to quantitate the numbers of both transfected viral producing cells and infected target cells, which is visible, direct and convenient. In addition to GFP, signal peptide FLAG was fused to the C-terminus of DAAO, which allows us to use anti-FLAG to detect the expression of the target protein without producing anti-DAAO antibody, which is tedious work and time consuming. Meanwhile, the function of the proteins were not impaired as shown by the properties and particular biological function of DAAO, positive FLAG antigen-antibody complex on Western blot analysis and positive green fluorescence under a microscope. The means of the vector delivery gene and the usage of tags above to detect target gene expression are useful for other gene therapies.

DAAO activation in the central nervous system is reportedly relevant to the incidence of schizophrenia (18,19), which had a potentially adverse effect on DAAO expression. Thus exposure endogenous DAAO to D-alanine may cause normal brain cell death and DAAO/D-alanine system may not be suitable to use in this group of patients. The different activities of endogenous DAAO and transfected DAAO, and the different reactions of D-alanine with endogenous DAAO and transfected DAAO need to be further studied.

In conclusion, in this study glioma 9L cells were introduced to the DAAO by recombinant retrovirus, then exposed to substrate D-alanine to cause cell oxidative stress and death. This demonstrated that the DAAO/D-alanine system has a potential utility for gene therapy and is an effective strategy for the treatment of brain cancer and other malignant tumors.

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