Over-expression of aldehyde dehydrogenase-2 protects against H$_2$O$_2$-induced oxidative damage and apoptosis in peripheral blood mononuclear cells

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Aim: To construct an eukaryotic expression vector containing the aldehyde dehydrogenase-2 (ALDH2) gene, and determine whether transfection with the ALDH2 gene can provide protection against hydrogen peroxide-induced oxidative damage, as well as attenuate apoptosis or cell death in human peripheral blood mononuclear cells (PBMCs).

Methods: The ALDH2 gene was cloned from human hepatocytes by RT-PCR. The eukaryotic expression vector containing the gene was constructed and then transfected into PBMCs via liposomes. RT-PCR, indirect immunofluorescence assay, and Western blot were used to evaluate the expression of the transgene in target cells. MTT assay and flow cytometry were used to detect the effects of ALDH2 on PBMCs damaged by hydrogen peroxide (H$_2$O$_2$). The level of intracellular reactive oxygen species (ROS) was determined by fluorescence spectrophotometry.

Results: The eukaryotic expression vector pcDNA3.1/myc-His-ALDH2 was successfully constructed and transfected into PBMCs. RT-PCR results showed higher mRNA expression of ALDH2 in the gene-transfected group than in the two control groups (empty vector-transfected group and negative control). Indirect immunofluorescence assay and Western blot indicated distinct higher protein expression of ALDH2 in the gene-transfected group. The cell survival rate against H$_2$O$_2$-induced oxidative damage was higher in the ALDH2 gene-transfected group. Moreover, apoptosis rates in gene-transfected PBMCs incubated with 50 and 75 μmol/L H$_2$O$_2$ decreased by 7% and 6%, respectively. The generation of intracellular ROS was also markedly downregulated.

Conclusion: ALDH2 gene transfection can protect PBMCs against H$_2$O$_2$-induced damage and attenuate apoptosis, accompanied with a downregulation of intracellular ROS. ALDH2 functions as a protector against oxidative stress.

Keywords: aldehyde dehydrogenase-2; oxidative damage; transfection; peripheral blood mononuclear cells; apoptosis

Introduction

The rapid development of medical and molecular biology techniques has resulted in the rise in importance of and focus on tumor immunotherapy research. In 1989, Komori[1] reported the first successful infusion of IL-2 activated donor lymphocytes after the recurrence of allogeneic hematopoietic stem cell transplantation (HSCT) for acute lymphoblastic leukemia. Since then, adoptive immunotherapy has continued to show challenging and novel application prospects in the treatment of malignant tumors. In recent years, great progress has been made in clinical tests involving adoptive immunotherapy, and various studies on phase I and II clinical trials have been conducted. Immune cells participate in immune response to protect the body during the therapeutic process, but as first-line cells, they are more vulnerable to oxidative damage[5-9]. In different diseases such as renal, cardiovascular, neoplastic, and neurodegenerative illnesses, as well as in aging[6-9], an oxidative stress-induced inflammatory event leading to cellular or tissue injury can be considered a unifying mechanism of injury. Reactive oxygen species (ROS), including hydroxyl radicals, superoxide anions, hydrogen peroxide (H$_2$O$_2$), and singlet oxygen, are generated not only in the process of radiotherapy or chemotherapy before HSCT, but also during therapy that uses drugs that cause DNA damage and induce subsequent apoptosis with the generation of ROS[10-12]. Excessive accumulation of ROS can induce oxidative damage to donor or recipient immune cells, weakening the effects of immunotherapy. The protection of immune cells against oxidative damage is, therefore, extremely important to
immunotherapy.

At present, many new therapeutic methods are being used in clinical treatments, and a recent strategy involves transferring protective genes in normal immune cells via appropriate vectors to enhance host immunological function against diseases. As a metabolic enzyme of toxic substances, mitochondrial aldehyde dehydrogenase (ALDH2) metabolizes acetaldehyde and plays a major role in the oxidation of acetaldehyde and protection against oxidative injury. It is highly expressed in human liver tissue and lowly expressed in peripheral blood mononuclear cells (PBMCs). It also varies with individual genetic diversity. Some people express inactive ALDH2 for gene mutation. ALDH2 is a nuclear-encoded mitochondrial enzyme that is localized in the mitochondrial matrix, and mitochondria are the major source of ROS. Excessive ROS attacks polyunsaturated fatty acids leading to membrane lipid peroxidation, thereby generating reactive aldehydes, including 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde. ALDH2 provides protection against oxidative damage through the oxidation of exogenous and endogenous aldehydes including 4-HNE, which is the product of lipid peroxidation[13–15]. Thus, ALDH2 gene modification may enhance anti-oxidative stress capacity of immune cells.

This research aims to determine whether the overexpression of ALDH2 can protect human PBMCs against H2O2-induced oxidative damage and attenuate apoptosis. We also investigated the level of ROS in this process. PBMCs are rich in immune cells, their activation and proliferation were studied in this research. This study is a convenient and effective initial foray into investigations on immune cells in gene therapy. Our team previously reported that the resistance to chemotherapeutic agents could be enhanced by transfection with resistant genes in human peripheral blood hematopoietic progenitor cells and PBMCs[16, 17]. In this study, we explored the protective effect of the ALDH2 gene and provide reference for further related research on gene therapy in practical immunotherapy.

Materials and methods

Construction of eukaryotic expression plasmid

Human ALDH2 cDNA (1.5 kb) was synthesized through reverse transcriptase polymerase chain reaction (RT-PCR) with total RNA extracted from normal human hepatocytes. The ALDH2 cDNA was cloned with DNA polymerase (TaKaRa, Japan) into pBS-T vector (TIANGEN, China) and sent to Jinsite Bio Inc (China) for sequencing. The primers used in PCR were ALDH2 forward 5'-GCTCGTCGTCGACAACGCGCTC-3' and ALDH2 reverse 5'-GACACGGATCCATGTTGCGCGCTGC-3'. To construct the eukaryotic expression plasmid expressing the ALDH2 gene pcDNA3.1/myc-His, plasmid (Invitrogen, USA) and pBS-T-ALDH2 were digested with restriction endonucleases EcoRI and BamHI, respectively. The refined ALDH2 segments and linearized pcDNA3.1/myc-His were ligated using T4 DNA ligase with a ratio of 2:1 to construct eukaryotic expression plasmid pcDNA3.1/myc-His-ALDH2 (pcDNA3.1(+)-ALDH2).

The eukaryotic expression plasmid was verified by PCR and digestion analysis.

Primary culture and activation of PBMCs

PBMCs from the blood of healthy adult volunteers were isolated using a Ficoll lymphocyte separating liquid, and cultivated in an RPMI-1640 culture medium (Gibco, USA) containing 10% fetal bovine serum in 6-well culture plates at 37 °C, 95% O2, and 5% CO2. Phytohemagglutinin (2.5 μg/mL) was added into the culture medium. After 8 h, the cells were moved into fresh medium, into which interleukin-2 (1000 U/mL) was added for activation. The culture medium was replaced with fresh medium 48 h later when the PMBCs were ready for use in the experiments.

Transfection of pcDNA3.1/myc-His-ALDH2 into PBMCs

For eukaryotic expression plasmid transduction, PBMCs were passaged into 6-well plates at a density of 1×10⁶ cells/well. When the cells reached 50% confluence (typically on the third day after subculturing), the medium was replaced with 1 mL fresh medium (without serum) containing 8 μL Lipofectamin 2000 transfection reagent (Invitrogen, USA) and 2 μg plasmid DNA. Cells in group 1 were transfected with pcDNA3.1/myc-His-ALDH2, and group 2 with pcDNA3.1/myc-His empty plasmid. Group 3 was used as the negative control group (without plasmid transfection). The medium was replaced with fresh medium after 4 h. Indirect immunofluorescence assay was used to observe the protein expression of ALDH2 after transfection. Transfection rate was calculated at 12, 24, 48, and 72 h according to the observed fluorescence intensity.

RT-PCR analysis

Total RNA was extracted using TRIzol isolation reagent (TaKaRa, Japan), and RT-PCR analysis was performed using a one-step RT-PCR kit (Bio Shiningene Inc., China). β-actin was used as internal control. The primers were actin forward 5'-GCTCGTCGTCGACAACGCGCTC-3' and actin reverse 5'-CAAAACATGATCTGGGTTGACATCTCTC-3'. RT-PCR analysis was performed at 42 °C for 60 min, 92 °C for 5 min, then at 30 cycles of 94 °C for 50 s, 70.5 °C for 60 s, 72 °C for 60 s, and 72 °C for 10 min. An aliquot (5 μL) from the RT-PCR product was subjected to electrophoresis in 1% agarose gel. The anticipated β-actin PCR product was 353 bp long and ALDH2 was 1576 bp.

Western blot analysis

For Western blot analysis, the cells were washed three times with PBS, homogenized in cell lysis buffer, incubated on ice for 20 min, and then centrifuged for 15 min at 10000×g. The aqueous supernatant was collected and quantified using a BCA protein assay kit (Boster, China). Equal amounts (20 μg) of protein extract were loaded and separated by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins on the gel were transferred to polyvinylidene difluoride membranes. The blotted membranes were blocked with 5% skim milk powder in TBS-buffer and then probed for 1.5 h.
with the ALDH2 monoclonal antibody (1:250; H0000217-M01, Abnova, Chinese Taipei) specific for human cellular ALDH2. After three 10-min washing with TBS-Tween 20 buffer (0.05%), the blots were incubated with a secondary antibody (1:3000; Goat Anti-Mouse IgG-HRP Conjugate secondary antibody, Abnova) for 1.5 h. Antibody binding was visualized using an enhanced chemiluminescence reagent (Santa Cruz, USA) according to the manufacturer’s instructions.

Cytotoxicity assay
The cytotoxicity of H₂O₂ to cells in groups 1–3 were examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test. Briefly, cells from the three groups were seeded into a 96-well cell culture plate at a density of 5×10³ cells/well and incubated overnight. The medium was discarded, and new medium containing 1000.000, 100.000, 10.000, 1.000, 0.100, 0.010, 0.001, and 0 μmol/L H₂O₂ were added into respective wells. Each concentration was tested four times. After 72 h, the medium was discarded and 20 μL of MTT (5 mg/mL) was added to the medium. After another 4 h, the medium was discarded through centrifugation, followed by the addition of DMSO to dissolve the endocellular crystal. OD value was measured at 570 nm on a microplate reader (B10 TEK EL×800UV, General Corporation, USA), with 485 nm as reference. Regression analysis was performed with SPSS followed by the calculation of IC₅₀ values.

Reactive oxygen species analysis
The measurement of intracellular ROS was based on the ROS-mediated conversion of non-fluorescent dichlorofluorescin diacetate (DCFH-DA) into dichlorofluorescin (DCFH). DCFH is a nonfluorescent compound that is oxidized into fluorescent 2, 7-dichlorofluorescin (DCF) in the presence of oxidants, which can be quantified using a fluorescence spectrophotometer. Briefly, 48 h after transfection, 1×10⁶ cells/well on the 6-well plates were harvested after incubation in the presence or absence of H₂O₂ for 8 h, and then replaced with serum-free medium containing 10 μmol/L DCFH-DA for 20 min at 37 °C. The cells were then rinsed three times with PBS. The fluorescence intensity of the cells was measured using a fluorescence spectrophotometer (Cary Elipse, USA).

Flow cytometric analysis of cell apoptosis
Seventy-two hours after transfection, transfection and expression of the transgene in target cells were evaluated and selected in the medium containing different concentrations of H₂O₂. For quantitative analysis of cell apoptosis, 1×10⁶ treated cells/tube in 500 μL binding buffer were incubated with Annexin V-FITC (5 μL) and propidium iodide (5 μL) for 5 min at room temperature. The ratio of apoptosis was analyzed by flow cytometry (FCM) (BD FACSCalibur, USA) with CellQuest research software.

Statistical analysis
Each experiment was performed at least three times. All values are expressed as means±SD. Data were collected by the Student’s t-test using SPSS13.0. P<0.05 was considered statistically significant.

Results
Construction of eukaryotic expression vector
Human ALDH2 was cloned from human hepatocytes by RT-PCR. The PCR optimum temperature was 70.5 °C (Figure 1A). The recombinant eukaryotic expression plasmid pcDNA3.1 (+)-ALDH2 was verified by PCR, sequencing, and endonuclease digestion. The sequencing results of recombinant plasmid showed that the cloned gene was identical to the ALDH2 cDNA published in GeneBank (NM_000690, sequence number Q0468). The fragment digested by endonuclease digestion analysis was about 1560 bp and the amplified fragment by PCR confirmation was 1576 bp, as expected (Figure 1B).

Figure 1. Detection of ALDH2 expression in normal human hepatocytes and recombinated plasmid pcDNA3.1(+)ALDH2. ALDH2 gene in human hepatocytes was cloned by RT-PCR. (A) Lane M: DNA Marker; Lanes 1–6: RT-PCR-amplified ALDH2 products in different PCR-extending temperatures, Lane 4 was 70.5 °C; Lane 7: negative control; ALDH2 eukaryotic expression vector was verified by PCR and endonuclease digestion; (B) Lane M: DNA marker; Lanes 1, 2: PCR-amplified ALDH2 products (1576 bp) of pcDNA3.1(+)ALDH2 vectors; Lanes 3, 4: results of endonuclease digestion; Lane 5: PCR-amplified ALDH2 products (1576 bp) of pcDNA3.1(+)ALDH2 vectors in bacterial liquid; Lane 6: DNA fragment of single enzyme digestion; Lanes 7, 8: circular DNA fragments of pcDNA3.1(+)ALDH2 and pcDNA3.1(+).
fluorescence remained bright for at least one week. Cells with weak fluorescence intensity was considered the negative control and those with the brightest density were as the positive control. The transfection efficiency was calculated. The transfection efficiency in PBMCs was (33.5±3.5)% at 24 h, (56.6±2.5)% at 48 h, and 100% at 72 h.

Expression of ALDH2 mRNA

The mRNA expression of ALDH2 in group 1 (transfected with pcDNA3.1(+)ALDH2) was considerably high, but there was no distinct ALDH2 band between groups 2 (transfected with pcDNA3.1/myc-His) and 3 (negative control group) (Figure 3). These results indicate that the transgene PBMCs had a high mRNA expression of ALDH2.

Expression of ALDH2 protein

Results of Western blot analysis demonstrated that the ALDH2-transfected cells in group 1 expressed a very high level of human ALDH2 protein compared with the empty vector-transfected group and the negative control group because the protein strips were distinct in Lane 1, but indistinct in the other two lanes (Figure 4). These results showed higher protein expression of ALDH2 in the gene-transfected group after being transfected for 72 h. PcDNA3.1/myc-His-ALDH2 was successfully expressed in PBMCs. These results indicate that the transfection of recombinant eukaryotic expression plasmid pcDNA3.1/myc-His-ALDH2 was an effective method for inducing overexpression of ALDH2 in cultured PBMCs.

Effect of ALDH2 on cultured PBMCs injured by H2O2

The relationship between the semilogarithm of H2O2 concentration and cell survival rate was determined by the MTT test (Figure 5). The IC50 of groups 1, 2, and 3 were obtained using the semilogarithmic regression equation (calculated with SPSS). The values were 8.833, 2.138, and 1.99 μmol/L, respectively. The semilogarithmic regression equation of groups 1–3

![Figure 2](image1.png)

**Figure 2.** ALDH2 protein expression in PBMCs by indirect immunofluorescence assay. Flourescence intensity of cells expressing ALDH2 proteins was observed 24 h after transfection. Cells in negative control group at 48 h (A) exhibited weak expression of ALDH2; cells in group 1 (pcDNA3.1(+)ALDH2 group) at 24 h (B); 48 h (C); and 72 h (D) exhibited considerably stronger fluorescence intensity (P<0.01, n=3). (Original magnification×20 NiKON E5400, F3.9, focal length: 14.5, ISO: 200).

![Figure 3](image2.png)

**Figure 3.** ALDH2 gene transfection induced over-expression of the mRNA of ALDH2 in PBMCs. Products of RT-PCR were analyzed by agarose gel electrophoresis. Lanes 1, 2, 3: RT-PCR amplified ALDH2 and β-actin products from experimental groups 1–3, respectively. β-actin was used as loading control. ALDH2 mRNA expression was considerably increased in cells transfected with pcDNA3.1(+)ALDH2, compared with that in un-transfected cells (P<0.01 vs control group, n=3).

![Figure 4](image3.png)

**Figure 4.** ALDH2 gene transfection induced over-expression of the protein of ALDH2 in PBMCs. ALDH2 protein expressions were detected by Western blot. β-actin was used as loading control. ALDH2 protein expression considerably increased in cells transfected with pcDNA3.1(+)ALDH2, compared with that in un-transfected cells (P<0.01 vs control group, n=3).

![Figure 5](image4.png)

**Figure 5.** Effect of ALDH2 on cultured PBMCs injured by H2O2. Cells were treated with different concentrations of H2O2. Semilogarithmic graphs of concentration-survival rate of PBMCs were drawn based on MTT results (group 1: pcDNA3.1(+)-ALDH2 group; group 2: pcDNA3.1 group; group 3: negative control) (P<0.05, n=4).
were $y=0.6261-0.1333 \ lg x$, $y=0.547-0.1424 \ lg x$, and $y=0.5413-0.1381 \ lg x$, respectively. The survival rate data indicated that ALDH2-modified PBMCs (group 1) had a higher tolerance for H$_2$O$_2$ than did groups 2 and 3 ($P<0.01$). There was no significant difference between groups 2 and 3.

**Effect of ALDH2 on ROS generation**

Treatment with 0, 50, 75, and 100 μmol/L H$_2$O$_2$ on PBMCs of the transgene group for 8 h produced a significant decrease in ROS in group 1 compared with that in groups 2 and 3 ($P<0.01$ or $P<0.05$; Figure 6A). There was no significant difference between groups 2 and 3 ($P>0.05$). ALDH2 transfection markedly decreased intracellular ROS generation, indicating that ALDH2 could prevent the increase of ROS generation induced by H$_2$O$_2$ at 50, 75, and 100 μmol/L (Figure 6B). ALDH2 transfection could decrease intracellular reactive oxygen species (ROS) generation within a certain concentration.

**Discussion**

In this study, we demonstrated that the overexpression of ALDH2 protected PBMCs against oxidative damage and considerably attenuated apoptosis. Oxidative damage could be induced by H$_2$O$_2$ and ALDH2 gene transfection enhanced the anti-oxidative effect accompanied with the decreased generation of intracellular ROS in PBMCs.

Adoptive immunotherapy is a new strategy for the treatment of tumors. PBMCs are rich in immune cells, which play a central role in immunotherapy, especially in adoptive immunotherapy. PBMCs are the source of various immune cells, such as natural killer (NK) cells and T lymphocytes. In fact, HSCT is being developed into a type of immunotherapy. Donor CD4', CD8', and NK cells have been reported to mediate graft-versus-leukemia effects. Oxidative stress is always induced in different diseases or the pretreatment of radiotherapy and chemotherapy before HSCT. However, immune cells are first subjected to oxidative damage through the generation of redundant ROS$^{18-24}$. Considering the improvements in genetic technology, the use of gene modification appears to be an attractive approach for the protection of immune cells against oxidative damage. We propose that ALDH2 could contribute to the protection of PBMCs against oxidative damage and interpret the mechanism of ALDH2 protection, as well as assist in immune therapy. We chose activated PBMCs, which provide abundant unseparated immune cells from healthy volunteers for the study; however, in clinical applications, we used isolated immune cells such as NK cells, which may have presented some limitations.

First, we cloned the ALDH2 gene from normal human hepatocytes and constructed the eukaryotic expression vector pcDNA3.1/mic-His-ALDH2, which was successfully transfected into PBMCs. The ALDH2 strongly affects alcohol metabolism. Inactive ALDH2 is considered contributory to alcohol flushing, preventing people from developing alcoholism$^{25-27}$. ALDH2 can also protect cells against damage induced by endogenous and exogenous toxic substances. Several reports have suggested that polymorphism ALDH2 genes have a fundamental relationship with colorectal cancer, primary hepatocellular carcinoma, and stomach cancer$^{28-31}$. More important, mitochondrial ALDH2 functions as a protector against oxidative stress$^{14, 32, 33}$. Our subsequent research verified this viewpoint.

As the inductor, H$_2$O$_2$ is a central oxygen metabolite during the complete reduction of oxygen to H$_2$O. It is a biologically important oxidant due to its ability to generate highly reactive and extremely potent hydroxyl radicals$^{34}$. The half life of H$_2$O$_2$ is longer than those of other reactive oxygen species; thus, it is typically used to induce oxidative stress in vitro$^{35}$. PBMCs are sensitive to H$_2$O$_2$-induced oxidative stress. In this study, the treatment of PBMCs with different concentrations of H$_2$O$_2$-induced apoptosis in a concentration-dependent manner. ALDH2 overexpression in PBMCs conferred cellular
resistance to H$_2$O$_2$ and considerably attenuated hyperoxia-induced cell apoptosis and death. It was proved that ALDH2 could protect PBMCs against H$_2$O$_2$-induced oxidative damage and apoptosis. The mechanism of these effects is attributed to the metabolism of 4-HNE and the downregulation of intracellular ROS.

In this study, the ROS production in cultured PBMCs was elevated after H$_2$O$_2$ exposure. Overexpression of ALDH2 decreased intracellular ROS production, indicating that ALDH2 might have antioxidant and cytoprotective effects on PBMCs. ROS are the by-products of normal cell metabolism during enzymatic electron-transporting processes, such as mitochondrial respiration, and there is an array of antioxidant systems for maintaining redox balance\cite{36}. Excessive accumulation of ROS, however, can result in the development of oxidative stress. The majority of ROS are produced in mitochondria, resulting in peroxidation of the mitochondrial membrane. The mitochondrial inner membrane is rich in polyunsaturated fatty acids, such as cardiolipin; thus, reactive aldehydes, including 4-HNE, would be easily derived from peroxidized polyunsaturated fatty acids. Thus, the rapid elimination of 4-HNE is necessary in mitochondria. Evidence supports the toxicity of 4-HNE, which results in injury and cell death through both apoptosis and necrosis\cite{37}. ALDH2 plays a major role in the oxidation of aliphatic and aromatic aldehydes, which are exogenous or endogenous, including 4-HNE. This study proved that the decrease in intracellular ROS is involved in the mechanism of protective effects, and mitochondrial ALDH2 plays a major role in the clearance of cytotoxic aldehydes derived from peroxides. Moreover, there may

Figure 7. ALDH2 gene transfection reduced cell apoptosis rate induced by H$_2$O$_2$. Annexin V and PI FACS analysis showed that early- and advanced-stage apoptotic rates decreased in group 1 (A). Treatment with ALDH2 gene transfection markedly decreased apoptosis compared with the two control groups. $n=3$. Mean±SD. *$P<0.05$, **$P<0.01$ vs control. (B) (group 1: pcDNA3.1(+)ALDH2 group; group 2: pcDNA3.1 group; group 3: negative control).
be other mechanisms related to these protective effects, such as the activation of Akt, AMP-activated protein kinase, and ERK/MAPK and PI3K-Akt pathways. However, further studies are still needed to shed light on these relationships\[14, 38\].

In summary, this study provides evidence that overexpression of ALDH2 can protect PBMCs against H$_2$O$_2$-induced oxidative damage and attenuate apoptosis. Our results also showed that compared with the control cells, the generation of intracellular ROS in gene-transfected cells markedly decreased after ALDH2 transfection. Mitochondrial ALDH2 functions as a protector against oxidative stress in PBMCs. Gene modification focusing on overexpression of ALDH2 and proteins in the immune cells may help elucidate the mechanism of the protective effect of ALDH2, and suggest potential novel gene therapies to assist in immunotherapy.

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Author contribution
Qin FANG, Ji-shi WANG designed the research; Xiu-ying HU performed the research; Jian-qiong XIE, Yuan YANG, Bai-qin FANG, Ji-shi WANG designed the research; Xiu-ying HU analyzed data and wrote the paper.

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