RESEARCH ARTICLE

Induction of Apoptosis in Human Leukemic Cell Lines by Diallyl Disulfide via Modulation of EGFR/ERK/PKM2 Signaling Pathways

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Abstract

**Background:** Diallyl disulfide (DADS) may exert potent anticancer action both *in vitro* and *in vivo*. Although its effects on cancer are significant, the underlying mechanisms remain unknown. In this study, we sought to elucidate possible links between DADS and pyruvate kinase (PKM2). **Materials and Methods:** KG1α, a leukemia cell line highly expressing PKM2 was used with a cell counting kit (CCK)-8 and flow cytometry (FCM) to investigate the effects of DADS. Relationships between PKM2 and DADS associated with phosphorylation of EGFR, ERK1/2 and MEK, were assessed by western blot analysis. **Results:** In KG1α cells highly expressing PKM2, we found that DADS could affect proliferation, apoptosis and EGFR/ERK/PKM2 signaling pathways, abrogating EGF-induced nuclear accumulation of PKM2. **Conclusions:** These results suggested that DADS suppressed the proliferation of KG1α cells, providing evidence that its proapoptotic effects are mediated through the inhibition of EGFR/ERK/PKM2 signaling pathways.

Keywords: Diallyl disulfide - leukemia cells - pyruvate kinase M2 - apoptosis - EGFR/ERK/PKM2 signaling pathways

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Introduction

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of immature white blood cells called “blasts”. The major interventions of conventional medicine failed to have its effects because of its side effects. Thus, it is urgent for us to find another effective therapy. An increasing number of researches have been focused on garlic, *Allium sativum*, a common plant used mainly as food and has recently been reported to have medicinal attributes, including antihypertensive, antiatherosclerotic and antioxidant properties (Bose, 2002; Block et al., 2007; Howard et al., 2007; Gayathri et al., 2009; Lee et al., 2011).

Epidemiological studies and laboratory experiments have recently demonstrated that sulfur-containing compounds, such as S-allyl cysteine, diallyl sulfide and diallyl disulfide (DADS), which contains two sulfur atoms and diallyl trisulfide, all of which are major components of garlic, may be associated with a reduced risk of certain cancers(Aggarwal and Shishodia, 2006; Antony, 2011). Among these, the biological activity of DADS, including its anticancer and anti-inflammatory effects, has been shown to be stronger (Bautista et al., 2005; Shin et al., 2010; Park, 2011; Tsubura, 2011; Park, 2012). In particular, this compound is known to inhibit the proliferation of various types of human cancer cells, through the induction of cell cycle arrest or apoptosis (Sundaram, 1996; Bottone, 2002; Kwon, 2002; Filomeni, 2003; Wen, 2004; Xiao, 2004; Arunkumar, 2007; Tan, 2008).

Studies of the unique metabolism of cancer began in the early 1920s when Otto Warburg proposed that tumors employ glycolysis, rather than the more efficient oxidative phosphorylation, for energy production. The consequences of this metabolic adjustment in cancer are higher glucose uptake and lactate secretion, features that have been termed the Warburg effect or aerobic glycolysis (Warburg, 1956). These observations have been debated ever since(Frezza, 2009). However, in the past decade, genetic studies of cancer predisposition syndromes and high-throughput sequencing of cancer genomes have revealed that mutations in metabolic enzymes make an important contribution to the etiology of the disease (Frezza et al., 2011). Consequently, the molecular basis of aerobic glycolysis in cancer has been biochemically investigated and a new era of cancer metabolism research has begun. This renewed interest in the field has revitalized a remarkable older claim that all cancer cells, independently of their tissue of origin, have increased levels of the glycolytic enzyme PKM2 isofrom (Mazurek et al., 2005). Pyruvate kinase (PK), which catalyzes the final step of glycolysis, has emerged as a potential regulator of this metabolic phenotype.
The M2 isoform of PK (PKM2) is highly expressed in cancer cells. Apart from its better-characterized cytosolic functions as a glycolytic enzyme, PKM2 has also been found in the nucleus. Nuclear translocation of PKM2 is possible due to a nuclear localization signal (NLS) in its C-terminal domain, which, in contrast to a classical NLS, is not rich in arginine and lysine (Hoshino et al., 2007; Setak et al., 2007). The proposed nuclear functions of PKM2 are varied. Nuclear PKM2 is required for cell proliferation after interleukin (IL)-3 stimulation and cell death after an apoptotic stimulus (Hoshino et al., 2007; Setak et al., 2007), however, the exact mechanisms behind these events are unclear. Other studies have proposed that nuclear PKM2 can interact with and activate transcription factors such as β-catenin, Oct-4, signal transducer and activator of transcription (Stat)3 and HIF-1, contributing to cell survival and proliferation (Lee et al., 2008; Luo et al., 2011; Yang et al., 2011; Gao et al., 2012). Epidermal growth factor (EGF) receptor activation induces nuclear translocation of PKM2 and c-SRC-mediating phosphorylation of β-catenin (Yang et al., 2011). Indeed, both PKM2 inhibitors have been designed in an attempt to halt tumor growth.

More recently, we found that diallyl disulfide, a characterized component of a Chinese herbal medicine with some demonstrated antitumor activities, was reported to inhibit PKM2 in the nucleus and induce cell death. We present evidence that DADS inhibits tumor cell proliferation, exerting its anti-cancer effects via EGFR/ERK/PKM2 pathways.

Materials and Methods

Cell culture

Human leukemia cell line K562, KG1α and HL-60 were kept in my own laboratory, and frozen as original stocks in 2009. Human monocyte was obtained from Healthy people. Human leukemia cells were cultured in Roswell Park Memorial Institute (RPMI-1640) medium containing 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Waltham, MA, USA) at 37°C, incubator at constant humidity cultured in cultures were maintained at 37°C in an air 5% CO₂ incubator at constant humidity.

Antibodies and chemicals

DADS was purchased from Adamsa Asset Management, was dissolved in Tween 80 (Sigma, USA) and adjusted to final concentrations using complete RPMI1640 (HyClone, USA). EGFR-specific inhibitor, AG-1478 was purchased from Sigma. Human peripheral lymphocyte (Hyclone, USA). EGFR-specific inhibitor, AG-1478 was added to each well and the cells were then incubated at 37°C for 2 h. Subsequently, plates were detected on a spectrophotometric plate reader (Shanghai Precision and Scientific Instrument Co., Ltd., Shanghai, China) at a wavelength of 450 nm.

Nuclear staining with Hoechst. Apoptosis of KG1α was observed morphologically by nuclear staining with Hoechst 33258 dye. Cells were spun onto glass slides by cytospin centrifuge, fixed with 4% paraformaldehyde for 10 min at 37°C, and incubated with 50μM Hoechst 33258 staining solution for 15 min in dark, then washed with PBS for t PBS and then blocked with 3% goat serum albumin. Cells were incubated with PKM2 antibody (1:100) in PBS overnight at 4°C. After three washes in PBS, slides were incubated for 1h in the dark with FITC-conjugated secondary goat anti-rabbit antibody (1:500). After three additional washes, slides were stained with PI for 30s to visualize the nuclei and examined using a Carl Zeiss confocal imaging system.

Reverse transcription polymerase chain reaction (RT-PCR) analysis. The total RNA from KG1α-cells was isolated with TRIzol and cDNA was generated using a High Capacity Invitrogen RT kit and an oligo (dT) primer. cDNA from with or without DADS treatment cell samples were amplified by quantitative Real-time PCR with specific primers for F-CCND1 (5'-GCATCTACACCGAACAATCCCATC-3') and R-CCND1 (3'-CGTAGATGTGGCTGTTGAGGTAG-5'), F-C-MYC (5'-GAGACAGATCAGCAACAACCGA-3') and R-c-Myc (3'-CTCCTGCTTGGGCTTGGGC-5'). β-actin gene was used as an endogenous reference to obtain relative expression values. The reaction mixture was carried out using 20 μg of template cDNA, 1X SYBR pre-mix EX Taq, and 0.5 μM forward and reverse primers in a final volume of 25 μl. Samples were amplified in the IQ SYBR Green PCR Master Mix (Bio-Rad, CA, U.S.A.) for 40 cycles under the following conditions: 95°C for 5 min, denaturing at 95°C for 20s, annealing at 55°C for 15s, and extension at 72°C for 30s. The efficiency of the target gene amplification was proven by examining the absolute value of the slope of log input amount versus ΔCT. Fold changes transcripts were calculated after normalization to endogenous β-actin, using comparative 2^ΔCT method calculated for each observed value where ΔCT was the difference in the observed CT values between the gene of interest and β-actin. The control group was set as 1. All procedures were repeated in triplicate.

Statistics analysis

The intensity of the immunoreactive bands was
Results

Inhibition of cell viability by DADS in KG1α cells

To examine the detect the expression levels of PKM2 protein in different leukemia cell lines, to find out the leukemia cell line (HL-60 cells, K562 cells, KG1α cells and mononuclear cells) which highly express PKM2. As shown in the results, KG1α cell is the leukemia cell line which highly expresses PKM2. In particular, since KG1α cells showed higher express PKM2 compared to HL-60 and K562 cells, later experiments were conducted with KG1α cells (Figure 1A). To examine the effects of DADS on the proliferation of KG1α cells, it was treated with appropriate concentrations of DADS for 24 h, 48 h and 72 h measured by cell counting kit-8 (CCK-8) assay. As shown in the results, as the concentration of DADS treatment increased, cell viability decreased in KG1α cells. For instance, when KG1α cells were treated with (50-300) μM of DADS, our results indicated the proliferation of cells was inhibited in a time and concentration-dependent manner after DADS treatment for 24 h, 48 h and 72 h (Figure 1B). The IC50, determined after 48 h DADS incubation, were 126.98 μM. Then KG1α cells grown under the same conditions were sampled and photographed under an inverted microscope (original magnification 400×) (Figure 1C).

Induction of apoptosis by DADS in KG1α cells

Next, we performed experiments to determine whether this inhibitory effect of DADS on KG1α cell growth resulted from apoptotic cell death. To examine apoptosis morphologically, we stained the nuclei of untreated and DADS-treated cells with Hoechst 33258. It is indicated that typical apoptosis morphological changes could be found in KG1α cells induced by DADS (Figure 2A). To investigate the alteration of cell cycle caused by DADS, the proportion of cells in each phases was measured with flow cytometric analysis. Furthermore, the percentages of cell cycle in G0/G1 phase in KG1α cells induced by DADS were (41.19±0.05)%, (54.32±0.02)% and (68.70±0.02)% respectively. The result that DADS could arrest the cell cycle in G0/G1 phase significantly (Figure 2B). The percentages of apoptotic cells after treatment by DADS at a concentration of 50,100μM for 48 h in KG1α cells were analyzed by flow cytometry. The percentages of early apoptosis in KG1α cells induced by DADS were (1.98±0.23)%, (17.75±0.80)% and (23.01±1.27)% respectively. The proportions of early apoptotic cells treated by DADS increased in a dose-dependent manner (Figure 2C). These results that DADS have inhibitory effect on KG1α cell growth.

Effects of DADS on the Expression of Apoptosis-Related Proteins and Cycle related proteins and mRNA in KG1α Cells. To identify the pathway involved in the apoptosis of the DADS-treated KG1α cells, we measured the protein expression of the death receptor-related, the Bcl-2, Bax, Caspase 3 by Western blotting. In the intracellular molecules related to the mechanism of intrinsic pathway apoptosis, Cleaved caspase-3, Bax, and Bcl-2 were used. Caspase exists in cells as an inactive precursor form. Apoptotic complex activates the inactive caspase to cleavage product showed at western blot analysis (Zou et al., 1997). Bax and Bcl-2 are other intracellular molecules associated with intrinsic pathway of apoptosis. In the Bcl-2 family members, Bax is in the proapoptotic family members and Bcl-2 is in the anti-apoptotic family (Ruvolo et al., 2001). In this study, compared to the control, in the low or high concentration group, the activity of caspase-3, Bax increased gradually, and Bcl-2 activity decreased. In addition, we measured the

Figure 1. Effects of DADS on Cell Viability in Human Leukemia Cancer Cells. (A) The cells (HL-60 cells, K562 cells KG1α cells and mononuclear cells) were detect the expression levels of PKM2 protein. (*p<0.05 vs. mononuclear cells; p<0.05 vs. HL-60) (B) The KG1α cells were treated with the indicated concentrations of DADS for 24 h, 48 h and 72 h. The cell viability was measured by the CCK-8 assay. Each point represents the mean ± SD of three independent experiments. The significance was determined by the Student’s t-test (*p<0.05 vs untreated control). (C) KG1α cells grown under the same conditions as (B) were sampled and photographed under an inverted microscope (original magnification 400×).
proteins and cyclin-related proteins, the p21 and cyclinD1 by Western blotting. CyclinD1 belongs to the cyclinD family. Cyclin D1 is required for the cell cycle G1/S transition. Over-expression of cyclin D1 is known to correlate with the risk of tumor progression. p21 is a cyclin-dependent kinase inhibitor. p21 bind to and inhibit the activity of cyclin-CDK2 or -CDK4 complexes, and thus function as a regulator of cell cycle progression at the G1 phase. In addition, c-Myc and CCND1 expression is known to be upregulated by PKM2. (Yang et al., 2012b).

In order to confirm the effects of DADS on antitumor, which has an effect on apoptosis-related proteins and cell cycle-related proteins, Western blot was performed with available antibodies (Anti-Bcl-2, anti-Bax, anti-Cleaved Caspase-3, anti-CyclinD1, anti-p21, anti-Bax and Cleaved Caspase-3) to determine the specific targets of DADS. When cells treated with 50 and 100 μM DADS for 48 h, there was a concentration-dependent decrease in levels of anti-apoptotic Bcl-2. In addition, the protein levels of Bax and Cleaved Caspase-3 were up-regulated by DADS in a concentration-dependent manner (Figure 3A). Moreover, low-level CCND1 and c-Myc mRNA expression were also found among DADS-treated KG1α cells compared with control group by real-time PCR (Figure 3B). These results suggested that DADS could significantly inhibit the proliferation of KG1α cell.

Decrease in the Levels of PKM2 in the nucleus and cytoplasm by DADS in KG1α Cells

PKM2 expression is upregulated in human cancer.
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Figure 5. Effects of DADS on the Levels of Proteins Involvemeng of the the EGFR /ERK/PKM2 Pathway in KG1α Cells. (A) KG1α cells were incubated with (0, 50,100μmol/L) DADS, after 8 h, p-EGFR, EGFR, p-ERK, ERK1, p-MEK1/2 and MEK were examined by Western blot (*p<0.05 vs untreated control, P-EGFR, P-ERK, P-MEK1/2, PIN1); (B,C) Cells under treatment with or without AG1478 (6.25μM) and DADS(100μM) treatment for an additional 8h. Cell lysates were prepared and subjected to Western blot for analysing the effects of DADS on expression of PKM2, EGFR, ERK1, MEK and phosphorylated forms. Nuclear LaminA/C and total cell lysates β-actin were used as controls. (*p<0.05 vs untreated control. PKM2, P-EGFR, P-ERK, P-MEK1/2, β-catenin)

Discussion

According to our previous studies demonstrate that ginseng polysaccharide effect of K562 leukemia cells, found that 306 differentially expressed genes, which of 220 genes increase, 86 genes decrease, glycolytic pathway of differentially expressed genes among them has: the Pyruvate Kinase II (Pyruvate Kinase M2, PKM2), glucose phosphate isomerase (PGI, Phosphoglucose isomerase, PGI), Hexokinase II (Hexokinase 2, Hk2), A lactic acid dehydrogenase (Lactate dehydrogenase, LDHA).The PKM2 involved in gene regulation and the metabolism of the tumor is becoming a hot spot of research. This experiment selected three kinds of leukemia cell lines and normal mononuclear cells, protein imprinting method, according to the results of PKM2 is highly expressed in leukemia cell lines, especially for
KG1α the highest levels of cells, so as to the research object experiment (Figure 1A).

Although findings from recent studies have demonstrated that DADS, a main organosulfur component found in garlic, can suppress the growth of various cultured human cancer cell lines in vitro, the biochemical mechanisms by which this compound exerts its actions remain unclear. In present study, we demonstrated that DADS could play as a potential anti-cancer medicine via abrogated EGF-induced nuclear accumulation of PKM2 and reduction EGFR/ERK/MEK signal pathway activity with a serial of assays. The capacity of various concentrations of DADS inhibited cell proliferation of KG1α leukemia cell in a dose and time-dependent manner (Figure 1B). Moreover, our results indicated morphological signs of apoptosis after treatment with DADS by inverted microscope (Figure 1A).

These antiproliferative activities were agreement with previous study that DADS has been suggested to inhibit human cancer cell growth (Sundaram, 1996; Lee et al., 2011). Apoptosis is the most common way that anti-tumor medicine induces cell death. To examine apoptosis morphologically, we stained the nuclei of untreated and DADS-treated cells with Hoechst 33258. It is indicated that typical apoptosis morphological changes could be found in KG1α cells induced by DADS (Figure 2A). Apoptosis analysis by flow cytometry showed after exposure to various concentrations of DADS for 48h, the percentages of early apoptosis (AV-positive and PI-negative) and the cell cycle in G0/G1 phase of KG1α cells were gradually increased (Figure 2B, C).

Cancer cell metabolism is exemplified by high glucose consumption and lactate production. Pyruvate kinase (PK), which catalyzes the final step of glycolysis, has emerged as a potential regulator of this metabolic phenotype. The M2 isoform of PK (PKM2) is highly expressed in cancer cells. However, the mechanisms by which PKM2 coordinates high energy requirements with high anabolic activities to support cancer cell proliferation are still not completely understood. Current research has elucidated novel regulatory mechanisms for PKM2, contributing to its important role in cancer. PKM2 is up-regulated in a variety of cancer cells. Here, we reported that DADS can decrease the expression of PKM2 in the nucleus at 8h (Figure 4A). These results were further supported by immunofluorescence analyses (Figure 4B).Epidermal growth factor (EGF) receptor activation induces nuclear translocation of PKM2, which is mediated by the ERK1/2-dependent phosphorylation of PKM2 S37 and consequently PIN1-catalized cis-trans isomerization of PKM2 for binding to importint5 (Yang et al., 2011). PKM2 directly regulates gene transcription. Within the nucleus, PKM2 binds to β-catenin and promotes its transcriptional activity. In particular, cyclin D1 and c-Myc expression is induced, which is required for EGF-induced cell proliferation (Yang et al., 2012c). As determined by Western blot and Quantitative real-time PCR, the expression of cycle related proteins and mRNA decreased after treatment with DADS for 48h (Figure 3A, B). It is showed that PKM2 was resistant to EGF-induced nuclear translocation and decrease regulate gene transcription by DADS. Using Western blot, we found that before the expression of Bax, Bcl-2 significantly differed between the drug group and control group in a whole-cell protein (Figure 3A), following DADS treatment for 8h, β-catenin decreased (Figure 5B), which might contribute to DADS induced apoptosis for down-regulation of β-catenin-mediated signal transduction pathway (Fan et al., 2013). Treatment with or without EGFR inhibitor AG1478 and DADS, which blocked EGF-induced phosphorylation of EGFR, ERK and MEK1/2, abrogated EGF-induced nuclear accumulation of PKM2. The results of Western blot displayed that DADS decreased the proteins level in KG1α cells (Figure 5).

In conclusion, DADS had obvious restraining effects on the proliferation of KG1α, it decrease the expression PKM2 to display effects. DADS can effect on EGF-induced phosphorylation of EGFR, ERK and MEK1/2. Such effects inhibited cell growth, proliferation and induce cell apoptosis through the suppression of the EGFR/ERK/PKM2 signaling pathway, abrogated EGF-induced nuclear accumulation of PKM2.

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