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Chk1, but not Chk2, is responsible for G2/M phase arrest induced by diallyl disulfide in human gastric cancer BGC823 cells

Su Bo [a,b,c,1], He Hui [a,b,1], Wang Li [a,b,1], Ling Hui [a,b], Xia Hong [a,b], Dong Lin [a,b], Dai Wen-Xiang [a], Wu You-Hua [a], Ai Xiao-Hong [a], Jiang Hao [a,*], Su Qi [a,b,*]

[a] Center for Gastric Cancer Research of Hunan Province, First Affiliated Hospital, University of South China, Hengyang, Hunan 421001, China
[b] Key Laboratory of Cancer Cellular and Molecular Pathology of Hunan Provincial University, Cancer Research Institute, University of South China, Hengyang, Hunan 421001, China
[c] Key Laboratory for Pharmacoproteomics of Hunan Provincial University, Institute of Pharmacy and Pharmacology, University of South China, Hengyang, Hunan 421001, China

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ABSTRACT

Diallyl disulfide (DADS) has been shown to cause G2/M phase cell cycle arrest in several human cancers. Here we demonstrate a mechanism by which DADS induces G2/M phase arrest in BGC823 human gastric cancer cells via Chk1. From cell cycle gene array results, we next confirmed that cyclin B1 expression was decreased by DADS, while the expression of p21, GADD45α and p53 were increased. Despite the lack of change in Chk1 gene expression in response to DADS according to the array analysis, intriguingly overexpression of Chk1, but not Chk2, exhibited increased accumulation in G2/M phase. Moreover, overexpression of Chk1 promoted the effect of DADS-induced G2/M arrest. Augmented phosphorylation of Chk1 by DADS was observed in Chk1-transfected cells, followed by downregulation of Cdc25C and cyclin B1 proteins. In contrast, phosphorylated Chk2 showed no obvious change in Chk2-transfected cells after DADS treatment. Furthermore, knockdown of Chk1 by siRNA partially abrogated DADS-induced downregulation of Cdc25C and cyclin B1 proteins and G2/M arrest. In contrast, knockdown of Chk2 did not show these effects. Therefore, these data indicate that DADS may specifically modulate Chk1 phosphorylation, and DADS-induced G2/M phase arrest in BGC823 cells could result in part from Chk1-mediated inhibition of the Cdc25C/cyclin B1 pathway.

1. Introduction

Gastric cancer is one of the most common cancers in the world, accounting for 8% of total cancer cases and 10% of total cancer-related deaths. In 2008, over 70% of new gastric cancer cases and deaths occurred in developing countries (Jemal et al., 2011). Although many anticancer drugs have been used for routine clinical treatment of patients with gastric cancer, the development of multi-drug resistance, toxicity and side effects are unfortunately common. Therefore, there is a pressing need for the development of effective new drugs with low toxicity.

Diallyl disulfide (DADS), a major organosulfur compound derived from garlic, exhibits multiple antitumor effects (Yi and Su, 2013). Numerous studies have shown that DADS induces G2/M phase cell cycle arrest in many tumors through various mechanisms. These mechanisms primarily involve the effects of DADS on the expressions, activities and interactions of cell cycle-associated regulators (Yi and Su, 2013). For examples, DADS arrests colon tumor cell lines in G2/M phase via inhibition of CDC2 kinase (cyclin-dependent kinase 1, CDK1) activity leading to decreased formation of CDC2/cyclin B1 complex (Knowles and Milner, 2000), increasing p21 expression by modulation of histone acetylation (Druesne et al., 2004), a p53-independent mechanism (Jo et al., 2008; Song et al., 2009). p21 is upregulated and PCNA (proliferating cell nuclear antigen) and cyclin B1 are downregulated by DADS in colon cancer SW480 cells (Liao et al., 2007, 2009). DADS induces G2/M arrest of liver cancer cells by controlling the expression of cyclin B1 and CDK7 (Wu et al., 2004). Downregulation of CDK1

Abbreviations: ATM, ataxia-telangiectasia mutated kinase; ATR, ATM-related protein kinase; Cdc 2, cell division cycle 2; CDK, cyclin-dependent kinase; Chk, checkpoint kinase; DADS, diallyl disulfide; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; Mcl1, myeloid cell leukemia sequence 1; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA; Wip1, wild-type p53-induced phosphatase 1.

* Corresponding authors. Tel.: +86 0734 8279150 (J. Hao). Address: Center for Gastric Cancer Research of Hunan Province, First Affiliated Hospital, University of South China, Hengyang, Hunan 421001, China. Tel./fax: +86 0734 8281547 (S. Qi).
E-mail addresses: jhao16859@163.com (J. Hao), suqi1945@163.com (S. Qi).
[1] These authors contributed equally to this work.

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expression is involved in DADS-induced G2/M blockage in prostate cancer PC-3 cells (Arunkumar et al., 2006). DADS increases cyclin E and decreases CDK2 expression in human bladder cancer T24 cells (Lu et al., 2004). DADS reduces the interaction of Mcl1 with PCNA and CDK1 in human leukemia HL-60 cells, leading to G2/M arrest (Yi et al., 2012).

Our previous research has shown that the growth inhibitory effect of DADS on human gastric cancer is due to induction of G_2/M phase arrest, which results in part from inhibition of ERK1/2 (Ling et al., 2006), increase of histone H3 and H4 acetylation accompanied by upregulation of p21 expression (Su et al., 2012), and decrease of Cdc25C protein expression via activation of the p38 MAP kinase pathway (Yuan et al., 2004). Nevertheless, the mechanisms of DADS-mediated G_2/M arrest in human gastric cancer cells remain to be further elucidated.

Checkpoint kinases 1 and 2 (Chk1 and Chk2) have emerged as critical mediators to transduce the checkpoint signal and facilitate cell cycle arrest and DNA repair. These kinases are activated by phosphorylation of upstream protein kinases in response to DNA damage by a variety of factors such as UV light, ionizing radiation, reactive oxygen species or DNA damaging chemotherapeutic agents (Reinhardt and Yaffe, 2009). The canonical ATR/Chk1 and ATM/Chk2 signaling pathways involved in cell cycle checkpoint control share common substrates of Cdc25 family protein phosphatases (Cdc25A, Cdc25B and Cdc25C). Chk1 and Chk2 phosphorylate and inhibit activities of Cdc25 proteins that act as positive regulators in driving cell cycle progression via conversion of the inactive hyperphosphorylated form of Cdk/cyclin complexes to the active state by dephosphorylation (Donzelli and Draetta, 2003). Chk1 and Chk2 have been proposed to be potential tumor suppressors, as their activation causes cell cycle arrest via their regulation of downstream effectors of cell cycle-associated proteins (Wang et al., 2008; Stolz et al., 2011). For instance, isocorydine-induced proliferation inhibition of hepatocellular carcinoma cells and G2/M phase arrest is involved in activation of Chk1 and inactivation of Cdc25C (Sun et al., 2012). Gallic acid inactivates Cdc25A/Cdc25C-cdc2 via ATM/Chk2 activation, resulting in cell cycle arrest, and induces apoptosis in human prostate carcinoma DU145 cells (Agarwal et al., 2006).

We previously demonstrated that DADS increases the phosphorylation of ATR and Chk1 and induces G_2/M arrest in gastric cancer BGC823 cells. However, DADS had no effect on p-Chk2 level (Ling et al., 2010). This suggests that DADS-induced G_2/M arrest may be mediated by the activation of Chk1 rather than Chk2. To evaluate this hypothesis, in the present study we first confirmed that DADS downregulated cyclin B1 but did not affect Chk1, Chk2 or Cdc25C gene expression at the transcriptional level using cell cycle gene arrays. The roles of Chk1 and Chk2 in DADS-induced G_2/M arrest were investigated by overexpression and knockdown analysis. The results indicated that Chk1 could dominantly modulate G_2/M checkpoint signaling, and DADS could induce G_2/M arrest through specific activation of Chk1, and decrease of Cdc5C and cyclin B1 protein levels.

2. Materials and methods

2.1. Reagents and antibodies

DADS (d420 = 1.0, Mr146.28, hallmark 80%), purchased from Fluka Co. (Milwaukee, WI, USA), was dissolved in Tween-80. The primary antibodies for phospho-Chk1 (sc-1792), phospho-Chk2 (sc-16297-R), Chk1 (sc-8408), Chk2 (sc-56296), p53 (sc-53394), p21 (sc-271610), GADD45α (sc-796), cyclin B1 (sc-245), and β-actin (sc-8432) and horseradish peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against Cdc25C was purchased from NeoMarkers (Lab Vision & NeoMarkers, Fremont, CA, USA).

2.2. Cell culture

Human gastric cancer BGC823 cells, established in the People's Hospital of Peking University, China, was provided by the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium (Gibco Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (Sijiqing Biological Engineering Material Co., Ltd., Hangzhou, China) with 100 U/mL penicillin and 100 U/mL streptomycin, and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Cell cycle analysis

BGC823 cells untreated or treated with 15 mg/L DADS were harvested and resuspended in 1 mL of ice-cold 75% ethanol and fixed for 24 h at 4 °C. After centrifugation, cells were resuspended in 1 mL of ice-cold 75% ethanol and fixed for 24 h at 4 °C. For subsequent flow cytometry analysis, fixed cells were washed with PBS once and resuspended in 1 mL of PI staining reagents (20 mg/L ribonuclease and 50 mg/L propidium iodide). After the samples were incubated in the dark for 30 min, the data were collected using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with Verity Winlist Software (Verity Software House, Topsham, ME, USA).

2.4. Cell cycle gene arrays

To identify the differential expression profiles of cell cycle-associated genes in DADS-treated BGC823 cells, we used human cell cycle SuperArray gene expression analysis kits (Super Array Co, Frederick, MD, USA). Briefly, total RNA was extracted using Trizol reagent after cells were treated or untreated by DADS for 24 h. The biotin-labeled cDNA synthesis and hybridization were performed according to the manufacturer's instructions. Hybridization signals on the array were detected using standard chemiluminescence procedures with ECL film (Hyperfilm ECL, Amersham Pharmacia Biotech, Uppsala, Sweden), and densitometric analysis of the images was conducted using ImagequantTM software (Amersham). All data were corrected for background intensities and normalized to housekeeping genes. The fold changes in gene expression were calculated between the untreated and DADS-treated groups. The genes showing significant differences by more than twofold were selected and are listed in Table 1.

2.5. Establishment of stable expressing Chk1 or Chk2 BGC823 cell line

To obtain recombinant Chk1 and Chk2 pcDNA3.1(+) vectors, full-length Chk1 and Chk2 were amplified from cDNA using primers including BamHI and HindIII restriction sites. The primer sequences were as follows: Chk1 F: 5′-CGG AAG CTT ATG GCA GTG CCC TTT G-3′, R: 5′-CGG CGA ATT CTC ATG TGG CAG GA-3′; Chk2 F: 5′-CGC CAA GCT TAT GTC TCG GGA GTC-3′, R: 5′-CGG AAT TCT CAC AAC ACA GCA GCA CAC-3′. Plasmids encoding Chk1 or Chk2 were constructed and transformed into *E. coli* DH5α. Positive colonies were selected and identified by PCR. The sequence and orientation of the Chk1 and Chk2 inserts were confirmed by restriction enzyme digestion and DNA sequencing. Cells were transfected with pcDNA3.1(+)/Chk1 or pcDNA3.1(+)/Chk2 using Lipofectamine 2000 from Invitrogen (Carlsbad, California, USA) according to the manufacturer's protocol. The stable transfectants were established after G418 (Invitrogen) selection. The expression levels of Chk1 and Chk2 in stable cell lines were evaluated by RT-PCR and western blot analysis.

Table 1
Differential expression genes in BGC823 cells treated by DADS for 24 h.

Gene	Accession no.	Treated/untreated
CDC45L	NM_003504	↑2.64
p21	NM_000389	↑2.24
CUL4A	NM_003589	↑2.47
GADD45α	NM_001924	↑2.24
RAD17	NM_002873	↑3.18
p53	NM_000546	↑3.32
ANAPC5	NM_016237	↓0.37
Cyclin A2	NM_001237	↓0.40
Cyclin B1	NM_031966	↓0.39
Cyclin E1	NM_001238	↓0.47
Cyclin E2	NM_004702	↓0.39
CDC16	NM_003903	↓0.03
CDC6	NM_001254	↓0.08
CDK5RAP1	NM_016408	↓0.35
GTF2H1	NM_005316	↓0.34
MCM5	NM_006739	↓0.05
RAD9A	NM_004584	↓0.31
RGC32	NM_014059	↓0.42