Abstract. Our previous study showed that sulforaphane (SFN) inhibits invasion in human prostate cancer DU145 cells; however, the underlying mechanisms were not profoundly investigated. In the present study, we found that sulforaphane-cysteine (SFN-Cys), as a metabolite of SFN, inhibits invasion and possesses a novel mechanism in prostate cancer DU145 and PC3 cells. The scratch and Transwell assays showed that SFN-Cys (15 µM) inhibited both migration and invasion, with cell morphological changes, such as cell shrinkage and pseudo-podia shortening. The cell proliferation (MTS) assay indicated that cell viability was markedly suppressed with increasing concentrations of SFN-Cys. Furthermore, the Transwell assay showed that inhibition of SFN-Cys-triggered invasion was tightly linked to the sustained extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. Western blot analysis revealed that SFN-Cys downregulated galectin-1 protein, an invasion-related protein, and that the galectin-1 reduction could be blocked by ERK1/2 inhibitor PD98059 (25 µM). Moreover, immunofluorescence staining showed that the expression level of galectin-1 protein was significantly reduced in the cells treated with SFN-Cys. Hence, SFN-Cys-inhibited invasion resulted from the sustained ERK1/2 phosphorylation and ERK1/2-triggered galectin-1 downregulation, suggesting that galectin-1 is a new SFN-Cys target inhibiting invasion apart from ERK1/2, in the treatment of prostate cancer.

Introduction

The mortality rate of prostate cancer has greatly increased globally in recent years, due to the fact that this type of tumor is not easily detected. Owing to its lack of noticeable symptoms, prostate cancer is commonly ignored. Once it is diagnosed, the tumor has usually invaded other tissues or organs and has undergone metastasis. It is essential to find a satisfactory strategy by which to treat prostate cancer, because to date traditional therapies applied including surgery, radiotherapy, chemotherapy and comprehensive therapy have not been effective in treating this disease. The reason is that these methods do not inhibit invasion and metastasis, thus the development of a new drug to suppress invasion and metastasis is the key for clinical treatment.

Epidemiological studies have shown that cruciferous vegetables reduce the risk of a variety of cancers (1). Sulforaphane (SFN), as an isothiocyanate, effectively inhibits the growth of various tumor cells (2). SFN metabolizes and generates metabolites such as sulforaphane-glutathione (SFN-GSH), sulforaphane-cysteine-glycine (SFN-CG), sulforaphane-cysteine (SFN-Cys), and sulforaphane-N-acetyl-cysteine (SFN-NAC). SFN metabolites may be the main compounds in tissues, rather than SFN (3). The metabolites, especially SFN-Cys, were found to inhibit histone deacetylase (HDAC) activity and have a higher plasma concentration and longer half-life (4,5), which may contribute to cancer inhibition. Our previous studies showed that SFN inhibited invasion through phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and CD44v6 downregulation in human prostate cancer DU145 cells (6). However, the key mechanisms of SFN-Cys in the inhibition of prostate cancer migration and invasion are not yet clear.

ERK1/2 are members of the mitogen-activated protein kinase (MAPK) family, which can be activated by various extracellular stimuli. ERK1/2 phosphorylation regulates the activation of downstream substrate molecules (7) and mediates signal transduction processes in many cancer cells. Sustained ERK1/2 phosphorylation was found to inhibit growth and invasion, and induce cell cycle arrest and apoptosis (8-11). Transient (<15 min) ERK1/2 phosphorylation was found to contribute to cancer proliferation and invasion (12,13). Further studies to find out the downstream signaling molecules are necessary for an overview of the whole signaling cascade.

Galectins are a β-galactoside-binding protein family, consisting of 15 members. It was reported that the expression level of galectin-1 is increased in various tumor cells.
including hepatocellular carcinoma (14), pancreatic ductal adenocarcinoma (15), oral squamous cell carcinoma (16), vulvar neoplasia (17) and colorectal cancer (18). Knockdown of galectin-1 through small interfering RNA in highly invasive cancer cells reduced invasion. Moreover, the invasion levels in poorly invasive cancer cells were significantly increased after overexpression of galectin-1 (19). Galectin-1 is involved in cell-to-cell, cell-to-extracellular matrix (ECM) adhesion and aggregation (20). Furthermore, transient activation of ERK1/2 contributed to galectin-1 increase, and the high expression of galectin-1 was reversed by the ERK1/2 inhibitor U0126 in T lymphocytes (21). Therefore, sustained ERK1/2 phosphorylation may lead to galectin-1 downregulation in human prostate cancer cells. The possible mechanisms of galectin-1 that contribute to cancer invasion need to be further investigated and discussed.

In the present study, we investigated the effects of SFN-Cys on prostate cancer cell proliferation, invasion and the underlying mechanisms, which will help us identify more targets and provide a basis for the clinical application of SFN-Cys in the treatment of prostate cancer.

Materials and methods

Reagents. D,L-sulforaphane-L-cysteine (SFN-Cys) and the anti-galectin-1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dimethyl sulfoxide (DMSO) was acquired from AppliChem GmbH (Darmstadt, Germany). RPMI-1640 culture medium was purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). β-actin antibody was purchased from ProteinTech Group, Inc. (Chicago, IL, USA). The phosphorylated ERK1/2 (pERK1/2), ERK1/2 and ERK1/2 inhibitor (PD98059) were obtained from Cell Signaling Technology, Inc. (Shanghai, China). The MTS assay kit was purchased from Promega (Madison, WI, USA). Transwell plates and Matrigel basement membrane matrix for invasion assay were obtained from BD Biosciences (Bedford, MA, USA). The DAPI staining solution was purchased from Beyotime Institute of Biotechnology (Nantong, China).

Cell culture. Human prostate cancer cell lines DU145 and PC3 were purchased from the Cell Resource Center, Peking Union Medical College (CRC/PUMC). Cells were cultured in RPMI-1640 medium with 10% FBS, 100 U/ml penicillin and streptomycin. The cells were maintained at 37°C in a humidified incubator containing 5% CO₂.

Cell morphology. DU145 and PC3 cells at 80% confluence were exposed to SFN-Cys at different concentrations (0, 5, 10, and 15 µM) for 24 h in 6-well plates. Cell morphology was observed with phase contrast microscope at x100 magnification (Leica, Germany). Digital cameras recorded the morphological change of the prostate cancer cells.

MTS assay. The cell viability was determined using the MTS assay kit (Promega). The cells (4x10⁴) were seeded in 96-well plates and treated with various doses of SFN-Cys for 24 h. Then 20 µl of MTS reagent was added to each well and incubated at 37°C for 1 h. The absorbance was measured at 490 nm on a BioTek Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA).

Scratch assay. The cells were cultured in 6-well plates for 10 h. Then, a 200-µl pipette tip was used to make two parallel wounds and one vertical wound per well. After being washed with PBS, the cells were incubated in serum-free medium at different doses of SFN-Cys for 24 h. The image of the wound area was captured by a phase-contrast microscope (Leica) at 0 and 24 h, and measured by the ImageJ processing program.

Invasion assay. The 24-well invasion chamber with 8-µm pores coated with Matrigel matrix was used for the cell invasion assay. Matrigel matrix was diluted with FBS-free medium to 2 mg/ml. The Transwell chambers were rehydrated with FBS-free medium at 37°C for 30 min, and then the cells (1x10⁵) were seeded in the upper chamber with 10% FBS culture medium. The 500 µl of culture medium was added to the lower chambers. After incubation at different doses of SFN-Cys for 24 h, the cells in the upper chamber were wiped off with cotton swab. The invaded cells in the lower chamber were fixed with 100% methanol for 20 min, and subsequently stained with 0.5% crystal violet solution for 20 min. Then, the cells were rinsed with distilled water and observed in five randomly selected fields per well under microscope. The ImageJ processing program was used for data analysis.

Immunoblotting. The cells were harvested and lysed with lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min. Then, the cell lysate was centrifuged at 12,000 x g for 10 min. The BCA protein assay kit (Invitrogen) was used to detect protein concentrations. Equal amounts of protein were separated using SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 1.5% BSA for 1 h. After incubation with primary antibodies overnight at 4°C, the fluorescence-labeled secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) was incubated with the membranes. After being washed, the protein bands were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences). β-actin was used as an internal control.

Immunofluorescence assays. The cells (4x10⁴) were seeded in a 24-well with glass coverslips and incubated for 10 h at 37°C. Following treatment with 15 µM SFN-Cys for 24 h, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 20 min at room temperature. After blocking through 5% BSA for 30 min, the cells were incubated with primary antibodies for 2 h and incubated with the fluorescence-labeled secondary antibody for 1 h. The glass coverslips were stained with DAPI and examined on confocal laser scanning microscope (Olympus FV1000; Olympus Corp., Tokyo, Japan).

Statistical analysis. The results are expressed as the mean ± SD, and analyzed using SPSS 18.0 software package by one-way ANOVA. The differences were considered statistically significant at p<0.05.
Results

SFN-Cys inhibits cell proliferation. MTS assay was used to assess the effect of SFN-Cys on cell viability. The cells were treated with 0, 5, 10, 15, 20, 25 and 30 µM SFN-Cys for 24 h. The results showed that cell viability was inhibited by SFN-Cys in a concentration-dependent manner (Fig. 1). Our study showed that 20 µM of SFN-Cys inhibited cell growth, but 15 µM of SFN-Cys did not markedly decrease cell viability. Thus, we chose 15 µM SFN-Cys as an optimal concentration for the invasion studies.

SFN-Cys inhibits cell invasion in the DU145 and PC3 cells. Transwell invasion assays were used to assess the effects of SFN-Cys on cell invasion. The cells were treated with different concentrations of SFN-Cys (0, 5, 10 and 15 µM). Then, the invaded cells were counted as described in Materials and methods. The results showed that cell invasiveness was significantly reduced when compared to the control group in a dose-dependent manner (Fig. 4A). Meanwhile, we aimed to ascertain whether SFN-Cys inhibits invasion via ERK1/2 activation. The ERK1/2 inhibitor PD98059 (25 µM) was added to the medium for 30 min, and then the cells were treated with 15 µM of SFN-Cys for 24 h. The results showed that the
invaded cells were significantly increased when compared to the SFN-Cys-only group in the DU145 and PC3 cells, respectively (Fig. 4B). These results suggest that SFN-Cys inhibited invasion via activation of ERK1/2 signaling in the human prostate cancer cells.

**SFN-Cys inhibits cell invasion of DU145 and PC3 cells via sustained ERK1/2 phosphorylation.** We further explored the molecular mechanisms involved in SFN-Cys-triggered invasion. Our previous studies showed that phosphorylation of ERK1/2 reached the highest degree at 24 h. Therefore, we chose 24 h as the optimal time for subsequent study. The cells were treated with increasing doses of SFN-Cys (0, 5, 10 and 15 μM) for 24 h. Western blot analysis showed that phosphorylation of ERK1/2 was significantly increased at 15 μM of SFN-Cys (Fig. 5). The results indicated that SFN-Cys inhibited invasion via activation of ERK1/2 in both the DU145 and PC3 cells.
SFN-Cys inhibits galectin-1-related invasion. Overexpression of galectin-1 promotes tumor cell invasion. To elucidate the mechanisms of SFN-Cys-induced invasion inhibition, we detected the expression of galectin-1 in the DU145 and PC3 cells. Immunofluorescence showed that galectin-1 was located in both the cytoplasm and the cell membrane. Galectin-1 expression was downregulated in the treated cells. Western blot analysis showed that galectin-1 expression was significantly decreased in the DU145 and PC3 cells. The results are expressed as the mean ± SD. *P<0.05 vs. the control, n=3. SFN-Cys, sulforaphane-cysteine.

SFN-Cys downregulates galectin-1 via activation of ERK1/2. We examined the link between ERK1/2 phosphorylation and galectin-1 expression. First, cells were treated with ERK1/2 inhibitor PD98059 (25 μM) for 30 min, then 15 μM of SFN-Cys was added to the medium for 24 h. Western blot analysis showed that phosphorylation of ERK1/2 was reduced with the increasing SFN-Cys concentrations (Fig. 6B). These results suggested that SFN-Cys inhibited invasion via downregulation of galectin-1 in the DU145 and PC3 cells.
markedly reduced, but downregulation of galectin-1 was reversed by PD98059 (Fig. 7), implying that galectin-1 is the downstream effector of ERK1/2 in the DU145 and PC3 cells. All the data indicated that SFN-Cys suppressed invasion via ERK1/2-mediated downregulation of galectin-1 in the human prostate cancer cells.

Discussion

SFN suppresses invasion in a variety of tumor cells (22-24). Due to a short half-life, SFN has not been used in clinical treatment. SFN-Cys, as a major metabolite of SFN, was found to have extensive tissue distribution in treated mice and a longer half-life (3,5). Therefore, it was more valuable to investigate the mechanisms involved in the inhibition of invasion in prostate cancer cells by SFN-Cys. In the present study, we found that SFN-Cys inhibited cell proliferation by MTS assay in the DU145 and PC3 cells. This provided an optimum concentration and treatment time with which to investigate invasion inhibition. Meanwhile, these data confirmed that SFN-Cys also inhibited tumor growth, which may be related to inhibition of cell proliferating signaling, such as transient activation of ERK1/2 and downstream oncoproteins. In our model, SFN-Cys triggered sustained activation of ERK1/2. These data triggered different results which include cell cycle arrest and apoptosis. More importantly, the phosphorylation of ERK1/2 also caused inhibition of invasion. We demonstrated that SFN‑Cys significantly suppressed invasion in the cells following treatment with 15 µM SFN-Cys by scratch and invasion assays, indicating that broccoli-derived SFN-Cys has anti-invasion potential in human prostate cancer cells.

In addition, we further explored the molecular mechanism of SFN-Cys-mediated inhibition of invasion. The ERK1/2 signaling pathway is associated with intracellular protein-protein interactions and the regulation of multiple cellular processes, such as proliferation, differentiation, invasion and apoptosis. It was reported that high expression of pERK1/2 is found in benign prostate lesions, suggesting a good prognosis (25). Moreover, activation of ERK1/2 inhibited invasion in various tumor cells. Our previous studies demonstrated that SFN inhibited invasion via persistent ERK1/2 phosphorylation in human glioblastoma cells (10) and prostate cancer cells (6). In this study, SFN-Cys significantly increased ERK1/2 phosphorylation in a dose-dependent manner, and effectively inhibited invasion in the DU145 and PC3 cells, which could be blocked by PD98059. These results indicated that SFN-Cys inhibited tumor invasion through sustained ERK1/2 activation in human prostate cancer cells.

Tumor invasion is a complex process, including adhesion and degradation of ECM, angiogenesis, and proliferation. Galectin-1 contributes to cell-to-ECM adhesion and migration (26). Studies have shown that galectin-1 promoted tumor invasion in oral cancer and lung adenocarcinoma (19). It was reported that galectin-1 expression is significantly correlated with tumor stage (27) and clinical prognosis (28). Our results showed that SFN-Cys markedly downregulated galectin-1 levels in the DU145 and PC3 cells. When the cells were treated with PD98059 and SFN-Cys, the downregulation of galectin-1 was reversed by PD98059. The immunofluorescence assays showed that galectin-1 was mainly located in the cytoplasm and the cell membrane of prostate cancer cells. These results demonstrated that galectin-1 inhibited tumor invasion through sustained ERK1/2 activation in human prostate cancer cells.
MMP-2, MMP-9 and MMP-14. As a result, the proteasome pathway may be a major player in the regulation of galectin-1 and tumor invasion; however further studies are needed.

Galectin-1, as a glycoprotein, plays roles in the cell membrane and the ECM. The carbohydrate chains of galectin-1 could interact with adhesion molecules, such as integrin and E-cadherin, on cell surfaces and in the ECM, regulating subsequent motility and adhesion (33). Studies have shown that galectin-1 stimulated collagen, fibronectin synthesis and laminin expression (16,34) that promoted cell-matrix adhesion. Furthermore, galectin-1 induced epithelial-to-mesenchymal transition (EMT) and upregulated integrins that mediated cell-ECM interactions (35). Upregulated galectin-1 was found to stimulate platelets to release angiogenesis-related factors (36). Furthermore, galectin-1 overexpression was found to cause chemoresistance and promoted carcinogenesis and invasion (37). In the present study, SFN-Cys significantly decreased galectin-1 expression, indicating that the use of SFN-Cys possesses a better chemotherapeutic effect. Meanwhile, SFN-Cys is absorbed and maintains appropriate blood and tissue concentrations (5), which suggests that SFN-Cys shows promise as an anticancer agent for clinical trial.

In summary, our results revealed that SFN-Cys inhibited invasion in human prostate cancer cells via persistent ERK1/2 phosphorylation which triggers galectin-1 downregulation. This study demonstrated that SFN-Cys has potential as an anticancer agent for prostate cancer therapy.

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References


