Sulforaphane Inhibits ICAM-1 Expression and Monocyte Adhesion in Human Bladder Cancer T24 Cells

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Abstract: Intracellular adhesion molecule-1 (ICAM-1) belongs to the immunoglobulin-like superfamily of adhesion molecules that mediate cell adhesion to other cells, and ICAM-1 is involved in cancer progression and recurrence. Since the ICAM-1 is considered as one of the therapeutic target against bladder cancer, we examined whether sulforaphane, an aliphatic isothiocyanate, could inhibit ICAM-1 expression in bladder cancer T24 cells. Sulforaphane inhibited phorbol 12-myristate 13-acetate (PMA)-induced ICAM-1 expression at the mRNA and protein levels in human bladder cancer cells, as revealed by reverse transcriptase polymerase chain reaction and western blot analyses, respectively. Specific inhibitor studies have shown that the transcription factors, activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB), are involved in PMA-induced ICAM-1 expression. We found that sulforaphane inhibited the activation of both AP-1 and NF-κB induced by PMA in bladder cancer cells. Interestingly, we also found that sulforaphane abrogated PMA-induced THP-1 monocyte adhesion to bladder cancer cells. Collectively, our results provide experimental evidence that sulforaphane could serve as a new therapeutic candidate against bladder cancer.

Keywords: Sulforaphane, Bladder cancer, ICAM, Adhesion

INTRODUCTION

Bladder cancer is the fourth and ninth most common cancer in men and women, respectively, in developed coun-tries [1]. Bladder cancer is also the second most prevalent neoplasm in men aged 60 years or above [2]. Interestingly, the recurrence rate of bladder cancer is the highest among all malignant tumors [3], and the high recurrence rates and ongoing invasive monitoring requirements make it the cancer with the highest lifetime cost per patient [4].

Bladder cancer has been classified into two types: non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). Approximately 70~75% of newly diagnosed bladder cancers are NMIBC, while 25~30% of tumors at initial diagnosis are MIBC [5]. NMIBC,
previously referred to as “superficial” bladder cancer, is found on the inner surface of the bladder. It tends to spread into the bladder lumen rather than into the bladder mucosa or muscle layers. NMIBC, with a 5-year survival rate of almost 90%, is usually treated by cystoscopic resection of the tumor, often followed by intravesical chemotherapy and/or immune therapy. Even though the mortality of NMIBC is low, because of its high probability of recurrence and the risk of ultimately converting into invasive disease (approximately 10%), it causes physical agony to patients and the cost of its therapy is high [6]. In contrast, MIBC has a markedly worse prognosis, with more than 50% of patients developing metastatic disease. The 5-year survival rate of such patients is only 6% [7-9]. The clinicopathologic characteristics of each stage of bladder cancer have been reported from numerous bladder cancer patients in the UK and India [10,11]. In case of NMIBC patients, various chemotherapy regimens have been developed to suppress recurrence after surgery. However, due to some side effects, a new moderate strategy is required. Previous studies have shown that vitamins, NSAIDS, EGFR inhibitors, soy isoflavones, and sulforaphane could inhibit bladder cancer recurrence [12-14].

Sulforaphane, i.e., 1-isothiocyanato-4-(methylsulfinyl) butane, is a phytochemical compound that includes the isothiocyanate group of organosulfur compounds [15]. Sulforaphane is one of the major phytochemicals found in several cruciferous vegetables, such as broccoli, cauliflower, Brussels sprouts, cabbage, and kale [16]. Although many epidemiological, in vivo, and in vitro studies have investigated sulforaphane-induced inhibition of bladder cancer, the mechanism of action is still unclear. This study suggests that sulforaphane inhibits the activation of the transcription factors, activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB), and consequently suppresses ICAM-1 expression, thereby inhibiting bladder cancer progression and recurrence.

MATERIALS AND METHODS

Chemicals

Sulforaphane (purity ≥98% by UPLC) was purchased from Millipore (MA, USA) and dissolved in dimethyl sulfoxide (DMSO). DMSO and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (MO, USA), and 4,6-diamidino-2-phenylindole (DAPI) was obtained from Thermo Fisher Scientific (MA, USA). AP-1 inhibitor (SR-11302) and NF-κB inhibitor (BAY-11-7082) were purchased from Tocris Bioscience (MO, USA) and Calbiochem (CA, USA), respectively.

Clinical data analysis and specimen collection

The de-identified bladder tumor specimens were obtained from Chonnam National University Hospital. The specimens were collected after approval from the institutional review board of Chonnam National University Hwasun Hospital (approval no. CNUH-06-070). ICAM-1 array data for comparison between superficial and infiltrating bladder cancers were obtained from the publicly available Stransky Bladder Statistics (www.researchgate.net/Bioinformatics_in_Cancer_Research).

Immunohistochemistry

A horseradish peroxidase (HRP)/3,30-diaminobenzidine-immunohistochemical staining method was used on formalin-fixed paraffin-embedded tissues. The 5 μm tissues sections were cut from each paraffin block and then mounted and dried on glass slides. Tissues were deparaffinized using xylene, then rehydrated in graded alcohol solutions and retrieved with retrieval buffer. Endogenous peroxidase activity was blocked with peroxidase-blocking solution (Dako, CA, USA), followed by incubation with rabbit anti-ICAM-1 (Abcam, MA, USA) overnight at 4°C. After washing in TBS-Tween20 buffer (TBST), tissues were stained using the DakoReal Envision HRP/diaminobenzidine detection system (Dako). Tissues were counterstained with hematoxylin and mounted with coverslips. Stained tissues were viewed and photographed using a light microscope.

Hematoxylin and eosin staining

Sections (5 μm thick) were cut from a formalin-fixed paraffin-embedded block for staining. The sections on slides were first steeped in xylene and then immersed in 100% and 90% ethanol successively. After washing with running water, the sections were incubated in hematoxylin solution for 5 min, followed by rinsing with running water again. The sections were then stained with eosin by incubating in eosin solution for 1 min, followed by washing with 95% ethanol, 100% ethanol, and 100% xylene successively.
Finally, the sections were mounted for observation under a microscope (AxioPlan 2; Zeiss, Germany).

**Cells and culture conditions**

Human bladder cancer T24 cells and human monocytic THP-1 cells were obtained from the American Type Culture Collection (VA, USA). T24 cells were cultured in DMEM (high glucose; HyClone, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in an atmosphere containing 5% CO2. THP-1 cells were cultured in RPMI medium (HyClone) supplemented with 10% fetal bovine serum and 0.05 mM 2-mercaptoethanol at 37°C in an atmosphere containing 5% CO2.

**Immunofluorescence staining**

Cultured cells were rinsed with phosphate-buffered saline (PBS), fixed in 3.7% paraformaldehyde for 10 min, treated with 0.1% Triton X-100 for 15 min to increase the permeability of the antibody, and then washed with PBS thrice. Non-specific binding was prevented by blocking with 1% goat serum for 1 h, following which the cells were incubated for 16 h at 4°C with rabbit anti-ICAM-1 antisera (1 : 400, Abcam, MA, USA) and later with Alexa Fluor 555-conjugated goat anti-rabbit IgG (1 : 1000, Invitrogen, CA, USA) secondary antibody for 1 h at room temperature. All nuclei were counterstained with 0.1 μg/mL of DAPI. Fluorescence images were acquired using a BX51 microscope (Olympus, Japan). Images were analyzed using UIS 2 Olympus camera Image Plus5.1 software (Olympus). Quantitative analysis was then performed using ImageJ software (version 1.50i).

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

Total RNA was extracted from T24 cells using TRIzol reagent (Invitrogen). Total RNA (1 μg) in each sample was used to synthesize cDNA with an M-MLV reverse transcriptase kit (Promega, WI, USA). The cDNA obtained was amplified using primer sets against genes encoding GAPDH and ICAM-1 using a PCR master kit (iNtRON, Gyeonggi-do, Korea). The sequences of the specific primers used for detecting ICAM-1 and GAPDH expression were: ICAM-1 forward, 5'-CGA CTG GAC GAG AGG GAT TG-3' and ICAM-1 reverse, 5'-TTA TGA CTG CGG CTG CTA CC-3' (289 bp); GAPDH sense, 5'-TTG TTG CCA TCA ATG ACC CC-3' and GAPDH antisense, 5'-TGA CAA AGT GGT CGT TGA GG-3' (836 bp).

**Western blot analysis**

After washing with PBS, T24 cells were detached with trypsin and stored at −80°C for further use. Total protein was extracted from T24 cells using PRO-PREP protein extraction solution (iNtRON) with a protease inhibitor mixture (aprotinin, leupeptin, pepstatin A, EDTA, and phenylmethanesulfonyl fluoride) following the manufacturer’s protocol. Total protein (30 μg) was separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon polyvinylidene fluoride membranes (Millipore). The membranes were blocked using a solution of 0.1% Tween-20 in Tris-buffered saline (TBST) containing 5% skimmed milk for 1 h and incubated with primary antibodies in TBST overnight at 4°C. The membrane was washed four times with TBST for 10 min intervals. Horseradish peroxidase-conjugated secondary antibody was used to detect immunoreactive proteins by chemiluminescence. The following primary antibodies were used: anti-phospho-c-fos, anti-phospho-c-Jun, and anti-phospho-IκBα. All the antibodies were purchased from Cell Signaling Technology (MA, USA). The total protein levels were assayed using the blotted membrane with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, MA, USA) for 30 min at 56°C and reprobing the membranes with rabbit polyclonal anti-c-jun, anti-c-fos, and anti-IκBα antibodies (Cell Signaling Technology).

**Promoter activity assay**

AP-1 and NF-κB promoter-driven reporter plasmids were purchased from Clontech (Palo Alto, CA, USA). T24 cells (3 × 10⁴ cells/well) were seeded onto 48-well plates and grown to 60~70% confluence, following which AP-1 promoter/NF-κB promoter-luciferase reporter plasmids (pGL3-AP-1; pGL3-NF-κB) and pRLTK were co-transfected into the cells using the FuGENE transfection reagent (Boehringer Mannheim, IN, USA). Cells were incubated in the transfection medium for 24 h; next, the medium was replaced with a 1% FBS-containing medium, and the cells were treated with PMA for 4 h after pretreating them with sulforaphane for 1 h.

**Adhesion assay**

T24 cells, grown in 96-well plates (1.5 × 10⁴ cells per
well-100 μL) were treated with PMA at 37°C for 24 h after 1 h of pretreatment with sulforaphane. THP-1 cells were labeled with 10 ng/mL calcein-AM (Sigma Aldrich Co., MD, USA) and incubated for 30 min at 37°C. Next, 4 × 10⁴ labeled cells were added to the T24 monolayers, grown in 96-well plates, to a final volume of 100 μL after washing twice with PBS and incubated in a CO₂ incubator for 1 h. Non-adherent cells were removed from the plate by gentle washing twice with PBS, and the fluorescence intensity of the monolayer-associated THP-1 cells was measured using a fluorescent plate reader. Calcein-AM is a cell-permeable, non-fluorescent, and hydrophobic compound, which is rapidly hydrolyzed by cytoplasmic esterases, releasing the membrane-impermeable, hydrophilic, and intensely fluorescent calcein. The fluorescence was excited at 488 nm using an argon laser, and the evoked emission was filtered with a 515 nm long pass filter using a fluorometer (Fluoroskan; Labsystems, Finland). The adhered cells were observed and photographed under a fluorescence microscope (Olympus).

RESULTS

ICAM-1 expression and bladder cancer progression

Since ICAM-1 is known to affect tumor proliferation and progression, we examined the expression of ICAM-1 in bladder cancer tissue. In the specimen section, we verified that the ICAM-1 was highly expressed at the mucosa area in the bladder cancer tissue (Fig. 1A). ICAM-1 expression data were obtained from the publicly available Stransky Bladder Statistics of RNA sequencing database and were analyzed by comparison between patients with infiltrative (32 samples) and superficial (25 samples) types of bladder cancer. The fold change was 4.49 with a p value of 7.86 × 10⁻⁷. We observed that the expression of ICAM-1 was higher in infiltrative bladder cancer than in superficial bladder cancer (Fig. 1B).

Sulforaphane inhibits PMA-induced ICAM-1 expression in T24 cells

Sulforaphane is a natural flavonoid that participates in several physiological processes, including the suppression of tumorigenesis. To examine the inhibitory effect of sulforaphane on PMA-induced increased ICAM-1 expression, sulforaphane-pretreated T24 cells were stimulated by PMA.

In preliminary experiments, PMA promoted ICAM expression in a concentration-dependent manner (0~200 nM, data not shown), and a concentration of 100 or 200 nM was used in this study. The results showed that the pretreatment of T24 cells with sulforaphane inhibited PMA-induced upregulation of ICAM-1 protein (Fig. 2A) and mRNA (Fig. 2B) expression in a concentration-dependent manner. The inhibitory effect of sulforaphane on ICAM-1 expression was further investigated by immunohistochemical staining in cells treated with PMA. As shown in Fig. 3, sulforaphane suppressed PMA-induced ICAM-1 expression in T24 cells. In the current study, sulforaphane (0~50 μM)-induced inhibition of PMA-stimulated ICAM-1 expression in human T24 cells by suppressing ICAM-1 transcription did not damage the viability of T24 cells (data not shown). These results conclusively support that sulforaphane has the biological function of inhibiting PMA-stimulated ICAM-1 expression in human T24 cells by suppressing ICAM-1 transcription.
Sulforaphane suppresses PMA-induced AP-1 expression in T24 cells

To elucidate the role of the transcription factor, AP-1, in the inhibition of PMA-induced upregulation of ICAM-1 expression by sulforaphane, T24 cells were treated with SR-11302 (SR), an AP-1 inhibitor. Treatment with SR decreased PMA-induced ICAM-1 expression at the mRNA level (Fig. 4A), suggesting that AP-1 is crucial for this expression. To investigate AP-1 promoter activity, T24 cells were transiently transfected with the AP-1 promoter-luciferase reporter construct and AP-1 promoter activity was then determined. As shown in Fig. 4B, treatment with sulforaphane inhibited AP-1 promoter activity in a dose-dependent manner. c-fos and c-jun are the elements of AP-1; therefore, we examined the effect of sulforaphane on the phosphorylation of c-fos and c-jun by performing western blotting to obtain further insights into the mechanism underlying sulfo-
Sulforaphane-mediated downregulation of AP-1 activity. Expectedly, sulforaphane abrogated the phosphorylation of both c-fos and c-jun (elements of AP-1) in a dose-dependent manner (Fig. 4C).

**Sulforaphane suppresses PMA-induced NF-κB activation in T24 cells**

The involvement of the transcription factor, NF-κB, in the inhibition of PAM-induced upregulation of ICAM-1 expression by sulforaphane was also determined in T24 cells. Cells pretreated with BAY-11-7082 (BAY), an NF-κB inhibitor, were treated with PMA, and the mRNA level of ICAM-1 was examined. As shown in Fig. 5A, treatment with BAY markedly decreased PMA-induced ICAM-1 expression. To examine the effect of sulforaphane on the transcriptional regulation of NF-κB, T24 cells were transiently transfected with an NF-κB promoter-luciferase reporter construct and the NF-κB promoter activity was determined. As shown in Fig. 5B, PMA-induced NF-κB promoter activity was decreased by sulforaphane in a dose-dependent manner. The active NF-κB whole complex is a heterodimer or homodimer containing NF-κB p65 and p50 or other related molecules. IκBs can segregate the active subunit of NF-κB, while the phosphorylation of IκB can lead to the translocation of the NF-κB subunit into the nucleus, thereby activating NF-κB, which is critical for target gene expression. To investigate the mechanism underlying sulforaphane-induced inhibition of NF-κB transcriptional activity, we performed western blotting to assess the phosphorylation of IκB. We observed that sulforaphane suppressed PMA-induced enhanced phosphorylation of IκB in a concentration-dependent manner (Fig. 5C).

**Sulforaphane suppresses PMA-induced cell adhesion of THP-1 to T24 cells**

The suppression of THP-1 cell adhesion to T24 cells by sulforaphane was examined using calcein-AM. Calcein-AM is a cell-permeable, non-fluorescent, and hydrophobic compound, which is rapidly hydrolyzed by cytoplasmic esterases, releasing the membrane-impermeable, hydrophilic, and intensely fluorescent calcein. As shown in Fig. 6A and B, pretreatment of T24 cells with sulforaphane decreased the number of adhesive THP-1 cells, indicating that sulforaphane reduces cell adhesion to T24 cells by downregulating ICAM-1 expression.

**DISCUSSION**

These results demonstrate that sulforaphane inhibits ICAM-1 expression in bladder cancer cells and may be considered a potential therapeutic agent for blocking cell-to-cell interactions. Over the years, sulforaphane, found in cruciferous vegetables, has been shown to have chemopreventive activity in vitro and in vivo. SFN protects cells from environmental carcinogens and also induces growth arrest and/or apoptosis in various cancer cells [17]. Many epidemiological studies have demonstrated the anti-cancer properties of sulforaphane in the bladder [14,18]. A prospective
A cohort epidemiological study conducted by the Health Professionals Follow-Up Study has shown that the consumption of cruciferous vegetables, particularly that of broccoli, reduces the risk of developing bladder cancer [19,20]. A previous study has reported a strong inverse association between bladder cancer risk and broccoli consumption; subjects who consumed ≥2 servings/week experienced a 39% lower risk, while those who consumed >1 serving/week experienced a 29% lower risk [21]. In a meta-analysis of ten clinical trials, cruciferous vegetable intake was associated with a lowered overall risk of bladder cancer [22]. The effects of sulforaphane on bladder cancer have also been extensively studied in vitro and in vivo. Recently, we also reported that sulforaphane could inhibit bladder cancer cell proliferation by suppressing HIF-mediated glycolysis under hypoxic conditions [23].

Several molecular mechanisms underlying the function of sulforaphane as a therapeutic and preventative agent against bladder cancer have been proposed. First, sulforaphane dysregulates carcinogenic metabolizing enzyme expression through Nrf2 and Keap1 regulation [24-26]. Keap1 is a sulfhydryl-rich protein that inhibits Nrf2 by promoting the ubiquitination of Nrf2 and subsequent proteasomal degradation [27]. However, the interaction of sulforaphane with Keap1 interferes with this function, increasing Nrf2 accumulation in the nucleus and activating its transcriptional activity. Sulforaphane may modulate the inhibition of phase I enzymes (e.g., cytochrome p450) and induce the expression of phase II enzymes (e.g., glutathione-S transferase, quinone reductase, and NAD (P)H-quinone oxidoreductase-1) [28-30]. Some phase I enzymes make carcinogenic compounds to become more hydrophilic through hydrolysis, oxidation, and reduction reactions [31,32]. In contrast, phase II enzymes conjugate with reactive intermediates, causing them to be water-soluble and then excreted via urine [31,32]. Second, sulforaphane modulates the cell cycle and induces the apoptosis of bladder cancer cells. Sulforaphane induces cell cycle arrest at the G2-M and S phases in invasive bladder cancer cells [33]. In addition, sulforaphane has been reported to induce caspase-8 and -9-mediated apoptosis in bladder cancer cells as well as cause the downregulation of Bcl-1 expression and upregulation of Bax expression [34]. Third, sulforaphane regulates several cancer-related genes. Gene regulation mediated by sulforaphane has been reported to be epigenetic and transcriptional. Accumulating evidence suggests that sulforaphane can regulate epigenetic activity through microRNA (regulation and function as a potent inhibitor of histone deacetylase and DNA methyl transferase [35]. In addition to epigenetic regulation, sulforaphane downregulates the expression of some genes that are important for cancer development and metastasis, such as ICAM-1 and COX-2, through transcriptional regulation [36]. In the present study, we demonstrated that sulforaphane inhibited PMA-induced ICAM-1 expression by suppressing AP-1 and NF-kB expression in T24 bladder cancer cells (Figs. 4 and 5).

ICAM-1, a member of the immunoglobulin superfamily, is a transmembrane glycoprotein cell surface receptor. It can be soluble or membrane-bound, and it participates in cell-to-matrix and cell-to-cell interactions [37]. ICAM-1 is known to affect tumor progression by affecting cell proliferation, angiogenesis, and metastasis. Interestingly, recent studies
have shown that ICAM-1 is expressed in tumor cells as well as bladder cancer cells, and cancer patients with high levels of ICAM-1 expression have poorer prognosis than healthy controls [38,39]. In addition, increased ICAM-1 expression correlates significantly with stage, depth of invasion, lymph node, and distant metastasis, indicating that ICAM-1 plays an important role in cancer progression and metastasis.

As shown in Fig. 1, high ICAM-1 expression leads to low survival rates in bladder cancer patients. For these reasons, the development of new chemotherapeutics that inhibit ICAM-1 expression has emerged as an important research concern. ICAM-1 mRNA expression is mainly regulated at the transcriptional level, and AP-1 and NF-κB are known major transcription factors.

This study demonstrated that sulforaphane inhibited the transcriptional activities of AP-1 and NF-κB induced by PMA, indicating that the inhibition of AP-1 and NF-κB expression is crucial for the suppression of ICAM-1 expression by sulforaphane in human bladder cancer T24 cells. Consistent with our results, previous studies [40,41] have reported that sulforaphane inhibits AP-1 expression in UVB-induced skin cancer, LPS-activated macrophages, and UVB-treated human keratinocytes. The active AP-1 complex may comprise a heterodimer between c-fos and c-jun [39]. ERK phosphorylation might be responsible for the regulation of c-fos activity, while JNK appears to activate c-jun. In the current study, sulforaphane treatment inhibited the phosphorylation of both c-fos and c-jun (Fig. 4). Although the inhibition of the phosphorylation of c-fos and c-jun is not clear, the inhibition of c-fos and c-jun phosphorylation may be due to the lowered phosphorylation. Recently, Subedi et al. [43] reported that sulforaphane is a single AP-1 inhibitor that lowers the activation of both c-jun and c-fos through JNK and ERK inhibition in LPS-activated microglial cells. This discrepancy is probably due to cell-specific response to sulforaphane or the differences in stimulation for gene expression.

In subsequent experiments, the role of NF-κB in the inhibitory effects of sulforaphane on PMA-induced ICAM-1 expression was also investigated. The following observations suggest that the inhibition of NF-κB by sulforaphane is involved in ICAM-1 gene regulation in T24 cells (Fig. 5): i) Sulforaphane treatment decreased NF-κB-dependent transcriptional activity and IκB phosphorylation; ii) ICAM-1 expression was inhibited by treatment with BAY, a specific NF-κB inhibitor. The exact mechanism underlying the inhibition of NF-κB expression by sulforaphane is unknown. Since sulforaphane is widely known as an antioxidant, it may be assumed that the inhibition of ROS production by sulforaphane may be involved [44].

In the present study, we found that sulforaphane could block ICAM-1 expression and adhesion between T24 bladder cancer and THP-1 cells (human acute monocytic leukemia cell line), suggesting that the sulforaphane-mediated downregulation of ICAM-1 expression is involved in decreasing cell adhesion. Even the contributions to human cancer are poorly understood, monocytes and macrophages are major components of the tumor microenvironment. In particular, monocytes are actively recruited to tumors, which they then infiltrate, and the direct contact between cancer cells and monocytes has been suggested to be a critical component of tumor progression [45].

Further studies are needed to clarify these mechanisms. Understanding the molecular mechanisms through which sulforaphane inhibits AP-1 and NF-κB activation and, in turn, prevents ICAM-1 expression will serve as a basis for designing more effective anti-cancer drugs.

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