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Sulforaphane Enhances MHC Class II-Restricted Presentation of Exogenous Antigens

Seulmee Shin[1,3], Ki-Sung Jung[2], Yoonhee Park[1], Young-Wook Ko[1], Chong-Kil Lee[2], Kyunghae Cho[3], Nam-Joo Ha and Kyungjae Kim[1,*]

[1]College of Pharmacy, Sahmyook University, Seoul 139-742,
[2]College of Pharmacy, Chungbuk National University, Cheongju 361-763,
[3]Department of Biology, Seoul Women's Universty, Seoul 139-774, Republic of Korea

Abstract

Sulforaphane is an isothiocyanate found in cruciferous vegetables that has been reported to be an effective cancer preventive agent inducing growth arrest and/or cell death in cancer cells of various organs. This paper reports that sulforaphane exerts immunomodulatory activity on the MHC-restricted antigen presenting function. Sulforaphane efficiently increased the class II-restricted presentation of an exogenous antigen, ovalbumin (OVA), in both dendritic cells (DCs) and peritoneal macrophages *in vitro.* The class II-restricted OVA presentation-enhancing activity of sulforaphane was also confirmed using mice that had been injected with sulforaphane followed by soluble OVA. On the other hand, sulforaphane did not affect the class I-restricted presentation of exogenous OVA at concentrations that increase the class II-restricted antigen presentation. At a high concentration (20 μM), sulforaphane inhibited the class I-restricted presentation of exogenous OVA. Sulforaphane did not affect the phagocytic activity of the DCs, and the cell surface expression of total H-2K^b, B7-1, B7-2 and CD54 molecules, even though it increased the expression of I-A^b molecules to a barely discernable level. These results show that sulforaphane increases the class II-restricted antigen presenting function preferentially, and might provide a novel insight into the mechanisms of the anti-cancer effects of sulforaphane.

Key Words: Sulforaphane, Dendritic cells, MHC class II, Antigen processing

INTRODUCTION

Sulforaphane (1-isothiocyanato-4-(methyl-sulfinyl)butane; CH_3-SO-(CH2)4-N=C=S) is the hydrolysis product of glucoraphanin, which is found at fairly high concentrations in cruciferous vegetables, such as broccoli (Posner *et al.*, 1994; Bones and Rossiter, 2006). Sulforaphane has attracted considerable attention as a multipotent anti-cancer agent because it modulates multiple cellular targets involved in cancer development including susceptibility to carcinogens, apoptotic death, cell cycle, invasion and metastasis (Zhang *et al.*, 1994; Gingras *et al.*, 2004; Pham *et al.*, 2004; Singh *et al.*, 2005; Myzak and Dashwood, 2006a; Thejass and Kutten, 2006; Jin *et al.*, 2007; Pledgie-Tracy *et al.*, 2007). The cancer-preventive activity of sulforaphane is due primarily to the induction of carcinogen-metabolizing enzymes, such as the phase II detoxification enzyme (Yang *et al.*, 1994; Maheo *et al.*, 1997). In addition, sulforaphane has direct cytotoxic effects on several cancer cells (Gamet-Payrastre *et al.*, 2000; Parnaud *et al.*, 2004; Herman-Antosiewicz *et al.*, 2006; Fimognari and Hrelia, 2007).

Emerging evidence has shown that sulforaphane also has a range of immunomodulatory activities. Sulforaphane was reported to enhance the natural killer (NK) cell activity and antibody-dependent cellular cytotoxicity (ADCC) in both normal and tumor-bearing mice (Thejass and Kutten, 2006b). The NK cell activating activity of sulforaphane has also been demonstrated in a transgenic mouse model of prostate cancer, TRAMP (transgenic adenocarcinoma of mouse prostate) (Singh *et al.*, 2009). The treatment of TRAMP mice with sulforaphane appeared to increase the production of IL-12 by DCs, which then activates NK cells (Singh *et al.*, 2009). Sulforaphane was also shown to enhance the antibody production to a T-cell dependent antigen, sheep red blood cells, in mice (Thejass and Kutten, 2007), and restore the age-related decrease in contact hypersensitivity and Th1 immunity by activating Nrf2-mediated antioxidant enzyme expression and glutathione synthesis (Kim *et al.*, 2008).

Since T cells can only recognize the antigens presented

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***Corresponding Author**
E-mail: kimkj@syu.ac.kr
Tel: +82-2-3399-1601, Fax: +82-2-3399-1617

on MHC molecules, modulators of the MHC-restricted antigen processing pathways could be novel pharmacological agents for regulating the T cell responses. Indeed, it was shown recently that cyclosporin A (CsA) and tacrolimus inhibit both the class I and class II MHC-restricted antigen presentation pathways in DCs, suggesting that the immunosuppressive activity of CsA and tacrolimus is at least in part due to inhibition of the antigen processing pathways (Lee *et al.*, 2005; Lee *et al.*, 2007). The macrophage-colony stimulating factor (M-CSF) was also shown to enhance the MHC-restricted antigen presentation (Han *et al.*, 2005). This suggests that modulation of the antigen-specific signal may be useful for therapeutic regulation of the T cell responses.

This paper provides evidence demonstrating that sulforaphane enhances MHC class II-restricted exogenous antigen presentation in professional antigen presenting cells (APCs). Sulforaphane, however, did not increase the MHC class I-restricted exogenous antigen presentation.

MATERIALS AND METHODS

Cells and reagents

The T cell hybridomas, CD8 OVA1.3 and DOBW, were kindly provided by Dr. Clifford V. Harding (Case Western Reserve University, Cleveland, OH) (Harding *et al.*, 1991; Harding and Song, 1994). The DC cell line, DC2.4, was obtained from Dana-Farber Cancer Institute, Boston, MA, USA (Shen *et al.*, 1997). Sulforaphane was purchased from LKT Laboratories (St. Paul, MN).

Generation of DCs from bone marrow cells

The total bone marrow cells obtained from the femurs of Balb/c or C57BL/6 mouse were cultured in a 6-well plate (5×10^6/well) in a culture medium supplemented with 400 U/ml recombinant mouse GM-CSF (CreaGene, Korea). At days 3 and 4 from the initiation of the culture, the nonadherent cells were discarded by replacing the culture medium with fresh medium containing the cytokines after gentle shaking. The DCs were harvested by gentle pipetting at day 5.

Preparation of peritoneal macrophages

Peritoneal macrophages were elicited by injecting 1 ml of 3% thioglycollate in sterile water into mouse peritoneum. After 4 days, the cells in the peritoneum were harvested by a peritoneal lavage with ice-cold PBS. The red blood cells in the cell preparation were lysed by treating the cells with an ACK lysis buffer (150 mM NH4Cl, 1 M $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4) for 1 min.

Preparation of OVA-nanospheres

Nanospheres containing OVA were prepared using a homogenization/solvent evaporation method, as described previously, with 400 μl of OVA-containing water (50 mg/ml OVA) and 2 ml of ethyl acetate containing Polylactic-Co-Glycolic Acid (PLGA, 100 mg/ml, Sigma-Aldrich) (Lee *et al.*, 2010). Fluorescein isothiocyanate (FITC)-containing PLGA-nanospheres were prepared by adding FITC to the ethyl acetate phase together with PLGA. The OVA content was determined using a micro-bicinchoninic acid assay kit (Pierce, Rockford, IL) after lysing the nanospheres in a lysis buffer containing 0.1% SDS and 0.1 N NaOH.

MHC class I-restricted presentation assay

The quantity of class I MHC-complexed OVA peptides on the DCs was assessed using CD8 OVA1.3 cells (Lee *et al.*, 2010). Briefly, the DCs (1×10^5/well) were incubated with the indicated amounts of sulforaphane for 3 h, followed by the addition of OVA-nanospheres (50 μg as OVA). After 2 h incubation at 37°C, the plate was washed twice with 300 μl/well of pre-warmed PBS and fixed with 100 μl/well of ice-cold 1.0% paraformaldehyde for 5 min at room temperature. The plate was washed three times with 300 μl/well of PBS. Class I MHC-complexed OVA peptide quantities were assessed by IL-2 secretion assays after culturing the paraformaldehyde-fixed DCs with CD8.OVA cells (2×10^4/well) for 18 h, as described previously (Lee *et al.*, 2010).

MHC class II-restricted presentation assay

Class II MHC-complexed OVA peptides quantities on the DCs were assessed using DOBW cells (Lee *et al.*, 2010). Briefly, the DCs (1×10^5/well) or peritoneal macrophages (1×10^5/well) were incubated with the indicated amounts of sulforaphane for 3 h, followed by the addition of OVA-nanospheres (50 μg as OVA). After 2 h incubation at 37°C, the unphagocytosed nanospheres were removed by suction and fixed with 100 μl/well of ice-cold 1.0% paraformaldehyde for 5 min at room temperature. Class II MHC-complexed OVA peptide quantities were assessed using IL-2 secretion assays after culturing the paraformaldehyde-fixed DCs with the DOBW cells (2×10^4/well) for 18 h, as described previously (Lee *et al.*, 2010).

Phagocytosis activity

DCs were added to the nanospheres containing both OVA and FITC (1 mg/well as OVA). After 20 min, the unphagocytosed nanospheres were removed by washing with pre-warmed PBS. The cells were then harvested and fixed in 1% paraformaldehyde in PBS. Flow cytometry analysis was then performed using a FACSCanto flow cytometer (BD Biosciences).

Phenotype analysis

The cells were stained with the monoclonal antibodies recognizing murine cell surface markers, as described previously (Lee *et al.*, 2001), and flow cytometry was then performed. The monoclonal antibodies, anti-H2-K^b, anti-I-A^b, anti-CD80, anti-CD86, anti-CD40, anti-CD54 and an isotype-matched control antibody were purchased from BD Biosciences.

Effects on IL-6 production

For BM-DCs, the cells (1×10^5/well) were treated with 100 nM lipopolysaccharide (LPS, Sigma-Aldrich) and different concentrations of sulforaphane for 5 h at 37°C. The culture medium containing LPS and sulforaphane was then sucked out, and added to fresh culture medium. After 18 h incubation, the culture supernatants were collected, and the amounts of IL-6 were measured using a commercial immunoassay kit (eBioscience, San Diego, CA). For the peritoneal macrophages, the cells (1×10^5/well) were pretreated with different concentrations of sulforaphane for 3 h, followed by the addition of OVA-nanospheres (50 μg as OVA). After incubation for 2 h at 37°C, the unphagocytosed OVA-nanospheres were removed by washing with PBS. After adding fresh culture medium, the plate was cultured for 18 h at 37°C. The amounts of

IL-6 in the culture supernatants were measured using a commercial immunoassay kit (eBioscience).

In vivo antigen presentation assay

Peritoneal macrophages were elicited by injecting 1 ml of 3% thioglycollate in PBS into a mouse peritoneum. After 4 days, sulforaphane (500 μg/kg) was injected subcutaneously. Two hours later, soluble OVA (2 mg/mouse) was injected into the peritoneum. Sulforaphane (250 μg/kg) was again injected subcutaneously 2 h after injecting the soluble OVA. The peritoneal macrophages were harvested from the peritoneum 2 h after the second injection of sulforaphane and washed. The class II MHC-complexed OVA peptide quantities were then assessed by IL-2 secretion assays after culturing the paraformaldehyde-fixed peritoneal macrophages with DOBW cells (1×10^5/well) for 18 h, as described previously (Lee *et al.*, 2010).

Statistical analysis

The statistical significance of the difference between the control and treatment groups was assessed using a one-way ANOVA followed by a Student's t-test.

RESULTS

Sulforaphane enhances the MHC class II-restricted presentation of exogenous OVA

The effects of sulforaphane on the class II presentation of exogenous OVA were first examined in BM-DCs generated from mouse bone marrow cells using GM-CSF. In this experiment, the BM-DCs were treated with the indicated amounts of sulforaphane for 3 h, and then allowed to phagocytose the OVA-nanospheres for 2 h. The unphagocytosed OVA-nanospheres and sulforaphane were then removed by washing with PBS. The BM-DCs were then fixed with paraformaldehyde, and the amount of MHC class II-restricted OVA peptide presentation was assessed using OVA-specific CD4 T cell hybridoma, DOBW cells. As shown in Fig. 1A, the treatment of BM-DCs with sulforaphane enhanced the MHC class II-restricted presentation of exogenous OVA dose dependently.

The effects of sulforaphane on the class II presentation of exogenous OVA were also examined in peritoneal macrophages isolated from the thioglycollate-elicited mouse peritoneum. The treatment of peritoneal macrophages with sulforaphane also increased the level of MHC class II MHC-restricted OVA peptide presentation (Fig. 1B).

Sulforaphane does not enhance the MHC class I-restricted presentation of exogenous OVA

The effects of sulforaphane on the MHC class I-restricted presentation of exogenous OVA were examined using a DC cell line, DC2.4 cells. In this experiment, the DC2.4 cells were treated with the indicated amounts of sulforaphane for 3 h, and allowed to phagocytose the OVA-nanospheres for 2 h. The unphagocytosed OVA-nanospheres and sulforaphane were then removed by washing with PBS. The cells were then fixed with paraformaldehyde. The amount of MHC class I-restricted OVA peptide presentation was assessed using OVA-specific CD8 T cell hybridoma, CD8 OVA1.3 cells. As shown in Fig. 2, treatment of DC 2.4 cells with sulforaphane (up to 5 μM) did not increase the MHC class I-restricted OVA peptide presentation significantly. At a high concentration (20 μM), sulforaphane inhibited the MHC class I-restricted presentation of exogenous OVA (Fig. 2).

Sulforaphane does not affect the phagocytic activity of DCs

To determine if the antigen presentation-increasing activity of sulforaphane was due to the enhancement of phagocytic activity, the BM-DCs were treated with different concentrations of sulforaphane for 3 h, and then incubated with the nanospheres containing both OVA and FITC for 2 h. The DCs were then washed with PBS, cooled on ice and harvested by gentle

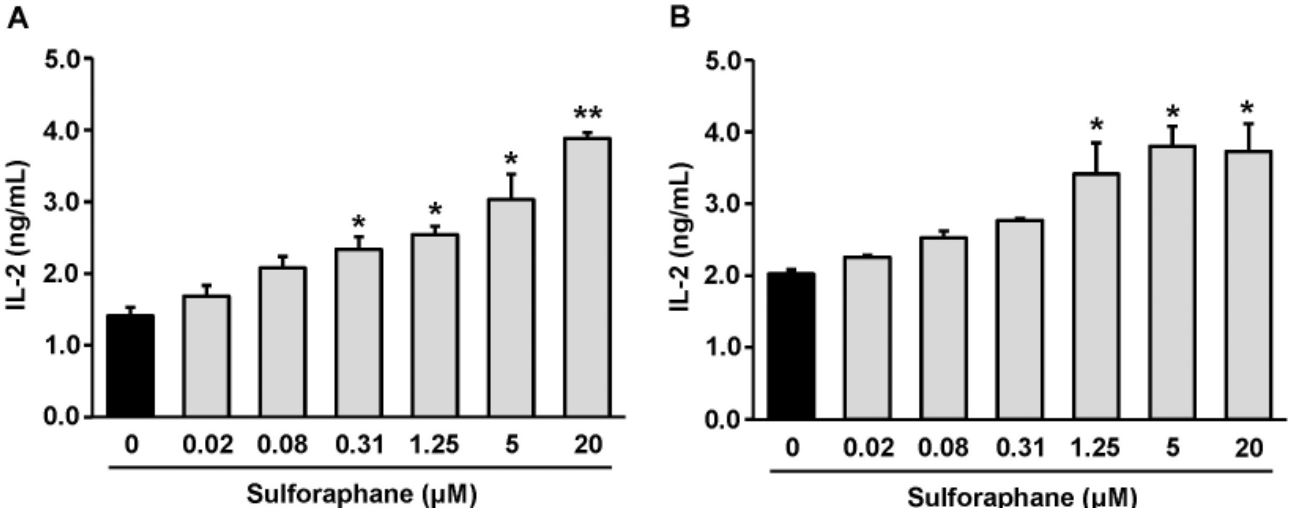

Fig. 1. Effects of sulforaphane on the MHC class II-restricted presentation of exogenous OVA. BM-DCs (A) and peritoneal macrophage (B) were cultured with the indicated amounts of sulforaphane for 4 h, followed by the addition of OVA-nanospheres (50 μg/ml as OVA). After 2 h incubation, the cells were washed and fixed. The amounts of I-A^b-OVA peptide complexes were assessed using DOBW cells. *$p < 0.05$, **$p < 0.01$ vs. untreated group.

pipetting. Flow cytometry of the harvested cells showed that the level of phagocytosis in the absence (shaded histograms) or presence (bold line histograms) of sulforaphane were similar. The results show that the MHC class II-restricted antigen presentation-increasing effect of sulforaphane is not due to the enhanced phagocytic activity of BM-DCs (Fig. 3).

Sulforaphane slightly increases the expression of MHC class II molecules

To determine if the MHC class II-restricted antigen presentation-increasing effect of sulforaphane was due to the enhanced expression of MHC molecules and co-stimulatory molecules on the cell surface, the DC2.4 cells were cultured with sulforaphane (20 μM) for 5 h, and the expression levels of MHC class I and class II molecules were measured by flow cytometry. As shown in Fig. 4, sulforaphane increased the expression of MHC class II (I-A^b) molecules slightly. Sulforaphane did not affect the expression of MHC class I (H-2K^b), B7-1, B7-2, and CD54 molecules to a discernable level (Fig. 4).

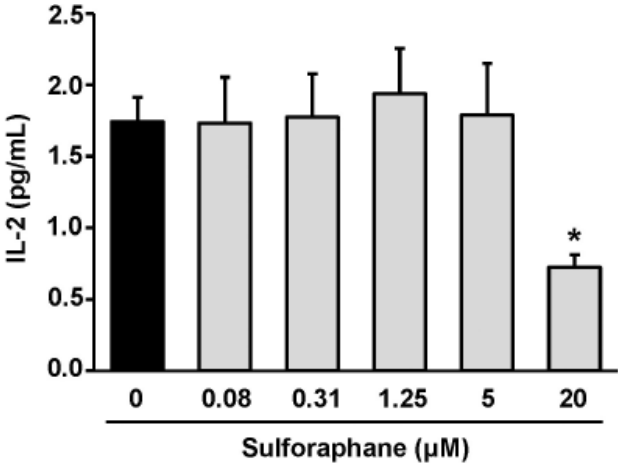

Fig. 2. Effects of sulforaphane on the MHC class I-restricted presentation of exogenous OVA. The DCs were cultured with the indicated amounts of sulforaphane for 4 h, followed by the addition of OVA-nanospheres (50 μg/ml as OVA). After 2 h incubation, the cells were washed and fixed. The amounts of K^b-OVA peptide complexes were assessed using CD8 OVA1.3 cells. *$p < 0.05$ vs. untreated group.

Sulforaphane inhibits cytokine production

The effects of sulforaphane on the production of cytokines in BM-DCs and peritoneal macrophages were examined to determine if the MHC class II-restricted antigen presentation-increasing effect of sulforaphane was due to the enhanced production of cytokines from APCs. In this experiment, BM-DCs and peritoneal macrophages were stimulated with LPS and OVA-nanospheres, respectively, in the presence of the indicated amounts of sulforaphane for 18 h. An assessment of the cytokines in the culture supernatants using commercial ELISA kits showed that sulforaphane inhibited the production of numerous cytokines, such as IL-6, IL-1 and TNF-α. Fig. 5 presents representative data showing that sulforaphane inhibited the production of IL-6. Sulforaphane inhibited the production of IL-6 in both LPS-stimulated BM-DCs (Fig. 5A) and OVA-nanosphere-stimulated peritoneal macrophages (Fig. 5B).

Sulforaphane increases the MHC class II-restricted exogenous antigen presentation *in vivo*

The *in vivo* relevance of the MHC class II-restricted antigen presentation-increasing effect of sulforaphane was examined in mice. In this experiment, peritoneal macrophages were first elicited by injecting thioglycollate into the mouse peritoneum. Four days later, sulforaphane was injected subcutaneously, and soluble OVA was then injected into the peritoneum. Peritoneal macrophages were harvested from the peritoneum 4 h after the soluble OVA injection and washed. The class II MHC-complexed OVA peptide quantities were assessed by IL-2 secretion assays using DOBW cells. As shown in Fig. 6, sulforaphane increased significantly the MHC class II-restricted OVA peptide presentation in peritoneal macrophages. These results show that sulforaphane enhances the MHC class II-restricted exogenous antigen presentation *in vivo.*

DISCUSSION

This study shows that short-term exposure to sulforaphane (6 h) increases MHC class II-restricted exogenous antigen presenting ability of professional APCs both *in vivo* and *in*

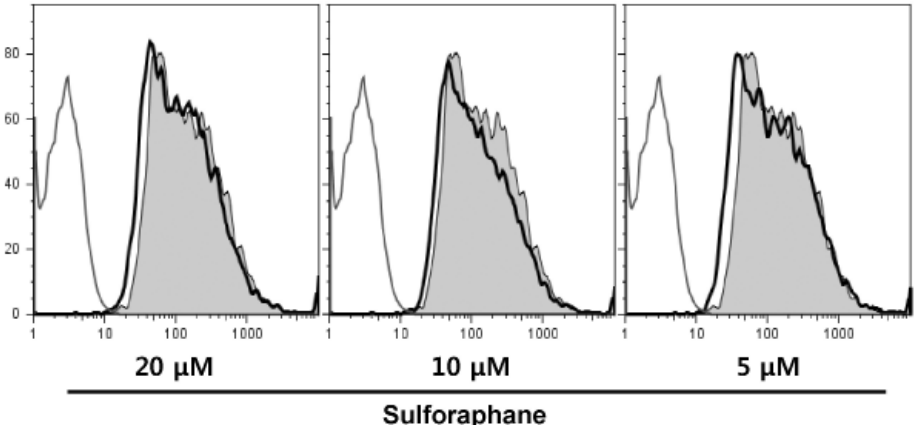

Fig. 3. Effects of sulforaphane on the phagocytic activity of BM-DCs. The BM-DCs were incubated with sulforaphane for 3 h, and the nanospheres containing both OVA and FITC were added. After 2 h, the cells were washed, harvested and analyzed by flow cytometry. Shade histograms represent the phagocytic activity of DCs in the absence of sulforaphane, and the bold lines represent the phagocytic activity of DCs in the presence of sulforaphane. DCs not incubated with the FITC-labeled nanospheres are shown as thin lines.

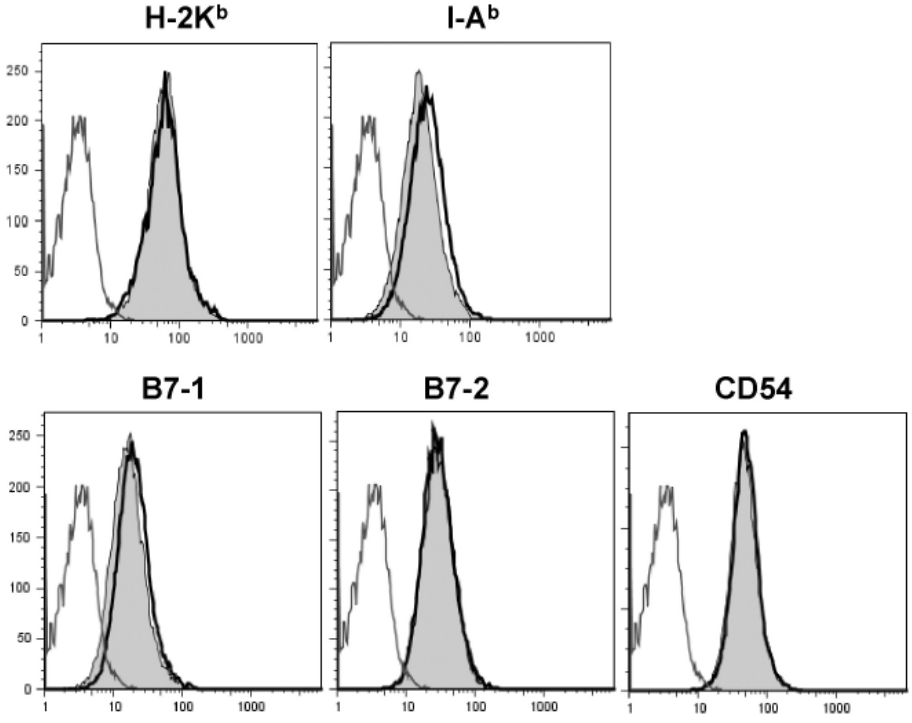

Fig. 4. Effects of sulforaphane on the expression of cell surface molecules. The DC2.4 cells were incubated with sulforaphane (10 and 20 μM) for 5 h, and harvested by gentle pipetting. The cells were then stained with the monoclonal antibodies recognizing murine H-2K^b, d I-A^b, B7-1, B7-2 and CD54) after blocking the FcR-binding anti-CD16/CD32 monoclonal antibody (clone 2.4G2). The shaded histograms represent the expression levels of the cell surface molecules in the absence of sulforaphane, and the thick line histograms represent the expression levels of those in the presence of sulforaphane.

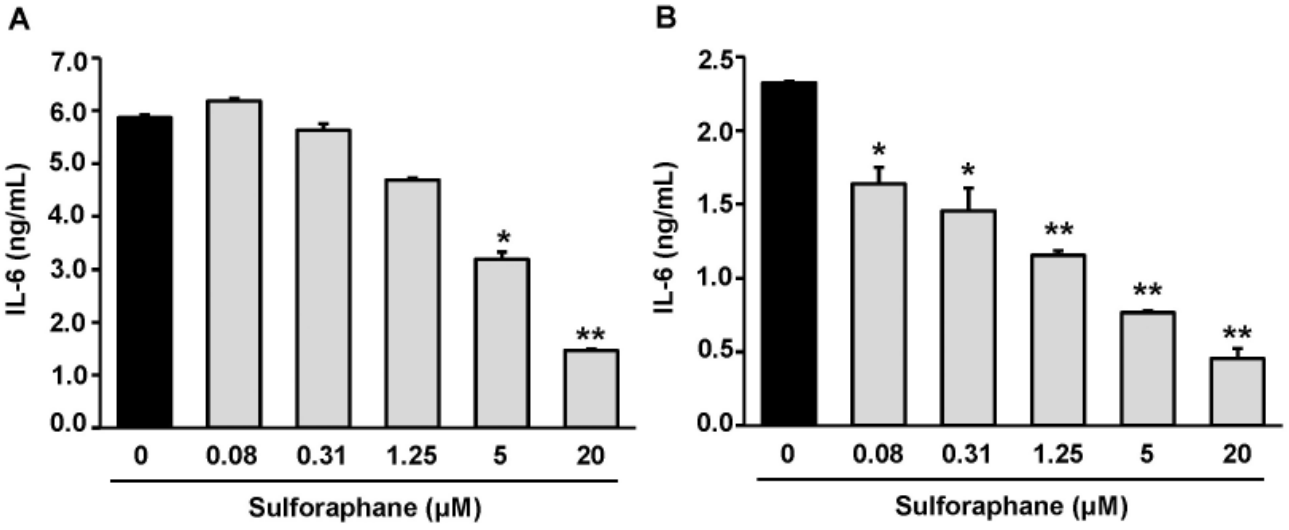

Fig. 5. Effects of sulforaphane on IL-6 production. BM-DCs (A) and peritoneal macrophages (B) were cultured in the presence of 100 nM LPS and OVA-nanospheres (50 μg as OVA), respectively, and indicated concentrations of sulforaphane for 5 h at 37°C. The cells were washed with PBS and fresh culture medium was added. After 18 h incubation, the culture supernatants were collected, and the amounts of IL-6 were measured using a commercial immunoassay kit. *$p < 0.05$, **$p<0.01$ vs. untreated group.

vitro. Sulforaphane did not affect the MHC class I-restricted exogenous antigen presenting ability of APCs. At a high concentration (20 μM), sulforaphane inhibited the MHC class I-restricted presentation of exogenous antigen. Since helper T cells can only recognize the antigens presented on MHC class II molecules (Guermonprez *et al.*, 2002), sulforaphane would affect many cellular immune reactions mediated by helper T cells or helper T cell products.

The classical paradigm of antigen presentation by professional APCs is that exogenous antigens are presented via MHC class II molecules to CD4$^+$ T cells, whereas endogenous antigens are presented via MHC class I molecules to CD8$^+$ T cells (Guermonprez *et al.*, 2002). In certain situations, professional APCs also present exogenous antigens via MHC class I molecules to CD8$^+$ T cells (Bevan, 1976; Harding, 1995). This process, which is termed cross-presentation, is an essential requirement for the induction of efficient CTL responses against viral or tumor antigens expressed in nonprofessional APCs (Huang *et al.*, 1994; Sigal *et al.*, 1999; Heath and Carbone, 2001). Since T cells can only recognize the antigens

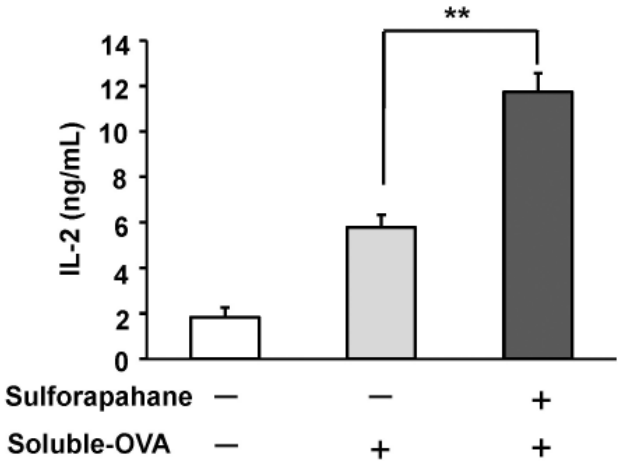

Fig. 6. Sulforaphane increases the MHC class II-restricted presentation of exogenous OVA in peritoneal macrophage *in vivo*. The peritoneal macrophages were elicited by injecting thioglycollate in PBS into the mouse peritoneum. After 4 days, sulforaphane (500 μg/kg) was injected subcutaneously. Two hours later, soluble OVA (2 mg/mouse) was injected into the peritoneum. Sulforaphane (250 μg/kg) was again injected subcutaneously 2 h after the injection of soluble OVA. The peritoneal macrophages were harvested from the peritoneum 2 h after the second injection of sulforaphane, washed, and then class II MHC-complexed OVA peptide quantities were then assessed by IL-2 secretion assays after culturing the paraformaldehyde-fixed peritoneal macrophages with DOBW cells for 18 h. ** p<0.01 vs. untreated group.

presented on MHC molecules (Guermonprez *et al.*, 2002), pharmacological agents activating the MHC-restricted antigen processing pathways might be useful for therapeutic immune activation. Sulforaphane is one of the compounds that were identified to increase the MHC-restricted exogenous antigen presentation in DCs. The mode of action of sulforaphane appeared to be peculiar in that it increases the class II-restricted exogenous antigen processing pathway, whereas it does not affect or even suppress the class I-restricted antigen processing pathways at high concentrations.

Sulforaphane reduces the rate of development and growth of tumors in animal models. Epidemiological studies have also shown that the dietary consumption of vegetables containing isothiocyanate compounds reduces the incidence of cancer development (Verhoeven *et al.*, 1996; Kolonel *et al.*, 2000; Ambrosone *et al.*, 2004). Sulforaphane exerts antitumor activity via the induction of carcinogen-metabolizing enzymes, such as phase II detoxification enzyme (Yang *et al.*, 1994; Maheo *et al.*, 1997). On the other hand, recent studies showed that sulforaphane exerts some of the beneficial effects via the activation of immune parameters. Sulforaphane enhances the natural killer (NK) cell activity and antibody-dependent cellular cytotoxicity (Thejass and Kutten, 2006b), increases the level of IL-12 production by DCs, which activates the NK cell activity (Singh *et al.*, 2009), enhances antibody production to a T-cell dependent antigen (Thejass and Kutten, 2007), and restores the age-related decrease in contact hypersensitivity and Th1 immunity (Kim *et al.*, 2008). This study adds another possible explanation for the beneficial effects of sulforaphane, i.e. the enhancement of exogenous antigen presentation to helper T cells. The activated helper T cells release cytokines and other stimulatory signals that stimulate the activity of macrophages, killer T cells and B cells (Zhu and Paul, 2008). The precise mechanisms by which sulforaphane increases the MHC class II-restricted antigen processing pathways remain to be determined. It is possible that sulforaphane activates the intracellular processing event of the phagocytosed antigens because sulforaphane did not enhance the phagocytic activity of DCs, nor the expression levels of MHC class II and major costimulatory molecules on DCs.

In all experiments, the DCs were exposed to sulforaphane for 4 h, and the cells were allowed to phagocytose OVA-microspheres for 2 h in the presence of sulforaphane. The DCs were then washed to remove the unphagocytosed OVA-microspheres, fixed with paraformaldehyde, and then washed thoroughly to remove paraformaldehyde before the functional assays with OVA-specific CD4 or CD8 T cells. Therefore, the activation of OVA-specific T cells must be due to the enhanced expression of OVA peptides on DCs, and not to the carryover of sulforaphane to the T cell cultures. OVA-specific T cell hybridomas such as DOBW cells and CD8 OVA1.3 cells were used to measure the amounts of OVA peptides complexed with MHC molecules. Therefore, the readouts are not quantitative measures of the amounts of OVA-derived peptide-MHC complexes. Nevertheless, this study clearly demonstrates that sulforaphane enhances the expression of OVA peptide-MHC complexes at sufficiently high levels on APCs to provoke OVA-specific T cell responses in professional APCs.

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