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Sulforaphane suppresses ultraviolet B-induced inflammation in HaCaT keratinocytes and HR-1 hairless mice☆

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Abstract

Ultraviolet B (UVB) irradiation induces skin damage and inflammation. One way to reduce the inflammation is via the use of molecules termed *photochemopreventive agents*. Sulforaphane (4-methylsulfinylbutyl isothiocyanate, SF), which is found in cruciferous vegetables, is known for its potent physiological properties. This study was designed to evaluate the effect of SF on skin inflammation *in vitro* and *in vivo*. In *in vitro* study using immortalized human keratinocytes (HaCaT), UVB caused marked inflammatory responses [i.e., decrease of HaCaT viability and increase of production of an inflammatory marker interleukin-6 (IL-6)]. SF recovered the cell proliferation and suppressed the IL-6 production. These anti-inflammatory effects of SF were explained by its ability to reduce UVB-induced inflammatory gene expressions [IL-6, IL-1β and cyclooxgenase-2 (COX-2)]. Because SF seems to have an impact on COX-2 expression, we focused on COX-2 and found that SF reduced UVB-induced COX-2 protein expression. In support of this, PGE_2 released from HaCaT was suppressed by SF. Western blot analysis revealed that SF inhibited p38, ERK and SAPK/JNK activation, indicating that the inhibition of mitogen-activated protein kinases (MAPK) by SF would attenuate the expression of inflammatory mediators (e.g., COX-2), thereby reducing inflammatory responses. Moreover, we conducted skin thickening assay using HR-1 hairless mice and found that UVB-induced skin thickness, COX-2 protein expression and hyperplasia were all suppressed by feeding SF to the mice. These results suggest that SF has a potential use as a compound for protection against UVB-induced skin inflammation.

Keywords: Cyclooxgenase-2; HaCaT; Inflammatory cytokine; Sulforaphane; UVB

1. Introduction

Cruciferous vegetables (e.g., broccoli, cabbage and kale) contain a variety of sulfur-containing compounds called glucosinolates, each of which yields a different isothiocyanate when enzymatically hydrolyzed [1]. For example, glucoraphanin (4-methylsulfinyl-butyl glucosinolate, GRP) is a predominant glucosinolate of broccoli (Fig. 1), and sulforaphane (4-methylsulfinylbutyl isothiocyanate, SF) is formed from GRP by myrosinase (thioglucosidase) when broccoli tissue is crushed or chewed [2]. Recently, vegetables that are rich in glucosinolates as well as isothiocyanates have received attention for their health benefits [3].

With regard to broccoli SF, in 1992, Zhang et al. [4] initially identified the compound as an inducer of quinone reductase, a Phase II detoxification enzyme. Subsequently, these authors reported that SF administration prevented dimethylbenz(*a*)-induced mammary tumors in rats [5]. At present, there are increasing evidences for such anticancer action of SF using cell cultures and/or animal models [6–8]. Besides the anticarcinogenic effect, biological studies suggest that broccoli SF has a wide variety of health benefits [9,10]. The beneficial effects of SF could be partly explained by its potent ability to regulate Phase II enzymes [11], to induce apoptosis [12], to inhibit angiogenesis [13] and to suppress inflammatory reactions [14]. In humans, a key factor in determining the efficacy of SF is in gaining an understanding of its absorption and metabolism. Some pharmacokinetic studies in both rats and humans reported that SF can be absorbed into blood and tissues [15–17]. SF reaches up to micromolar levels in blood plasma.

On the other hand, the skin is prone to inflammation, since the skin, which covers the entire body, is constantly exposed to sunlight stress such as ultraviolet (UV) light irradiation. UVB component (290–320 nm) is believed to be the major cause of a variety of cutaneous

Abbreviations: COX-2, cyclooxgenase-2; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GRP, 4-methylsulfinyl-butyl glucosinolate; HaCaT, immortalized human keratinocytes; IL, interleukin; MAPK, mitogen-activated protein kinases; PGE_2, prostaglandin E_2; RT-PCR, reverse transcriptase-PCR; SF, 4-methylsulfinylbutyl isothiocyanate; UV, ultraviolet.

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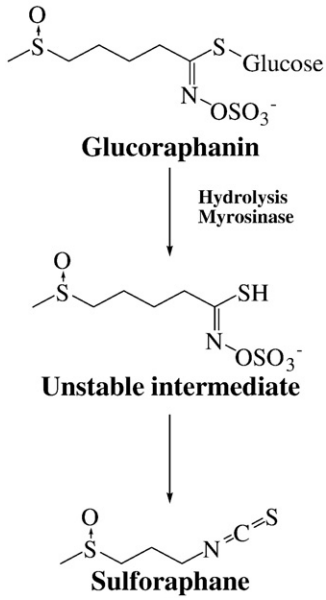

Fig. 1. SF is an aglycone (breakdown product) of glucosinolate GRP. Enzyme myrosinase transforms GRP into SF.

disorders [18,19]. Indeed, UVB induces skin erythema within several hours after exposure and, in extreme cases, causes edema. These symptoms are the so-called sunburn reaction, and severe sunburn reaction is involved in the development of skin cancer as well as in the promotion of skin aging [20]. Consequently, there are considerable works being undertaken to screen potential compounds that can protect human skin inflammation against UV damage. Dietary constituents including epigallocatechin gallate [21] and resveratrol [22] have been reported to prevent UV-induced skin disorders *in vitro* and/or *in vivo*.

On the basis of this background, in this study, we evaluated the effect of SF on skin inflammation in cell culture study and found that SF suppressed UVB-induced skin inflammation. In the latter part of this study, the inhibitory mechanism and the *in vivo* efficacy of SF were investigated. At present, although there have been many reports on the health benefits of broccoli SF or GRP [9–14,23], none of these has provided any information about the effect on skin inflammation except for the study by Talalay et al. [14]. Therefore, our findings (inhibitory effect of SF on skin inflammation with a new mechanism) would provide a novel insight into the application of SF as well as of cruciferous vegetables for anti-photoinflammatory purposes.

2. Materials and methods

2.1. Materials

SF was purchased from LKT Laboratories (St. Paul, MN, USA). GRP was extracted from broccoli sprout and purified using chromatography [16]. WST-1 reagent was obtained from Dojindo Laboratories (Kumamoto, Japan). All other reagents were of analytical grade.

Table 1
Primer sequences for cDNA amplification of selected human genes

Gene	GenBank accession no.	Sequence
il-1β	NM_003376	Forward 5′-CCAGGGACAGGATATGGAGCA-3′ Reverse 5′TTCAACACGCAGGACAGGTACAG-3′
il-6	NM_000584	Forward 5′-AAGCCAGAGCTGTGCAGATGAGTA-3′ Reverse 5′TGTCCTGCAGCCACTGGTTC-3′
prgs2/ cox-2	NM_000963	Forward 5′-TGAGCATCTACGGTTTGCTG-3′ Reverse 5′-TGCTTGTCTGGAACAACTGC-3′
actb/ β-actin	NM_001101	Forward 5′-TGGCACCCAGCACAATGAA-3′ Reverse 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′

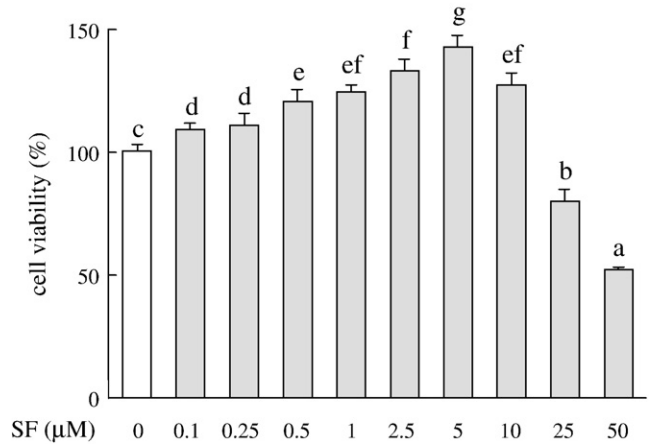

Fig. 2. Effect of SF on HaCaT proliferation under non-UVB irradiation condition. HaCaT cells (1×10^4 cells) were preincubated in the growth medium (DMEM containing 10% FBS) for 24 h. Then, the medium was changed to the test medium (DMEM containing 0.1% FBS supplemented with or without SF), and incubation was performed for 24 h. The viable cells were then assessed by WST-1. Cell viability data is expressed as the percentage to control. Values are mean±S.D. (n=6). Means without a common letter differ; P<.05.

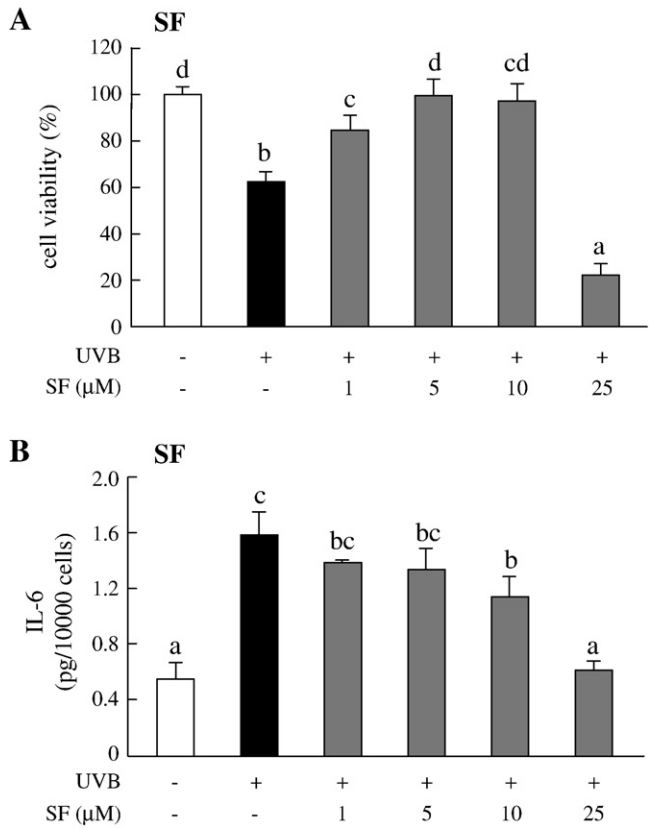

Fig. 3. Effect of SF on HaCaT proliferation (A) and IL-6 secretion (B) under UVB irradiation condition. HaCaT cells (1×10^4 cells/well) were preincubated in the growth medium (DMEM containing 10% FBS) for 24 h. Then, the medium was changed to the test medium (DMEM containing 0.1% FBS supplemented with or without SF), and incubation was performed for 24 h. After being washed with PBS, cells in PBS were exposed to UVB (50 mJ/cm^2). The UVB nontreated cells were also prepared as negative control. These UVB-treated or nontreated cells were cultivated in sample-free fresh test medium (DMEM containing 0.1% FBS) for 24 h. After that, the viable cells were assessed by WST-1 (A), and aliquot of the conditioned medium was used to measure the levels of IL-6 by ELISA (B). Cell viability data is expressed as the percentage to negative control. IL-6 data is expressed as secretion of IL-6 pg/10,000 cells. Values are mean±S.D. (n=6 for cell viability; n=3 for IL-6 secretion). Means without a common letter differ; P<.05.

2.2. Cells and animals

An immortalized human keratinocyte cell line (HaCaT) [24] was supplied by Prof. Norimichi Nakahata (Graduate School of Pharmaceutical Sciences, Tohoku University, Japan). HaCaT cells were cultured in a growth medium [Dulbecco's Modified Eagle's Medium (DMEM), D6046; Sigma, St. Louis, MO, USA], supplemented with 10% fetal bovine serum (FBS) (Dainippon Pharmaceutical, Osaka, Japan), 100 kU/L penicillin and streptomycin (100 mg/L) (Gibco BRL Rockville, MD, USA) at 37°C in 5% CO_2/95% air atmosphere in a humidified incubator. Female HR-1 hairless mice (6 weeks of age) were obtained from Hoshino Experimental Animal Center (Yashio, Japan) and were housed in cages kept at 23°C with a 12-h light/dark cycle in pathogen-free condition. The mice were acclimatized with laboratory rodent chow (MF; Oriental Yeast, Tokyo, Japan) and water for 1 week prior to the study.

2.3. Preparation of SF or GRP for cell cultures

SF was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM. The solution was diluted with test medium (DMEM containing 0.1% FBS) to achieve the desired final concentration (0–25 μM). The final concentration of DMSO in the test medium was less than 0.1% (v/v), which did not affect cell viability. SF-free test medium was prepared and used as control for the study.

2.4. Sample treatment and UVB irradiation

HaCaT cells (1×10^4 cells/well for cell proliferation and enzyme-linked immunosorbent assay, $8–10\times10^5$ for analysis of mRNA expression and $4–8\times10^6$ for Western blot analysis) were preincubated in the growth medium (DMEM containing 10% FBS) for 24 h. Then, the medium was changed to the test medium (DMEM containing 0.1% FBS supplemented with or without SF), and incubation was performed for 24 h. After being washed with PBS, cells in PBS were exposed to UVB (50 mJ/cm^2) delivered from an EL lamp [8 W, UVL-28, 302/365 nm, 100 V (UVP); Upland, CA, USA] that emits an energy spectrum with high fluence in the UVB region with a peak at 302 nm. The emitted UVB dose was regularly measured with an UV Light Meter UV-340 (Lutron, Coopersburg, PA, USA). The UVB nontreated cells were also prepared as negative control.

2.5. Cell proliferation

Proliferation was evaluated by WST-1 assay [25]. WST-1 is a tetrazolium salt that is converted into soluble formazan salt by succinate-tetrazolium reductase in the respiratory chain of active mitochondria of proliferating viable cells. The amount of formazan produced is directly proportional to the number of viable cells. WST-1 assay was conducted in test medium without SF to exclude the effect of these compounds. The UVB-treated or nontreated cells were cultivated in sample-free fresh test medium (DMEM containing 0.1% FBS) for 24 h, and WST-1 solution was then added to each well followed by incubation at 37°C for 3 h. Cell proliferation was determined by measuring the absorbance (450/655 nm) of the medium using a microplate reader (Model 550, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.6. Enzyme-linked immunosorbent assay

The UVB-treated or nontreated cells were cultivated in sample-free test medium for 24 h, and then an aliquot of the conditioned medium was used to measure the levels of interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2), using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The results were normalized to the number of cells per well.

2.7. Isolation of total RNA and analysis of mRNA expression

The UVB-treated or nontreated cells were cultivated in sample-free test medium for 3 and 6 h. From the cells, total RNA was isolated with RNeasy plus Mini kit (Qiagen, Valencia, CA, USA) for real-time quantitative reverse transcriptase-PCR (RT-PCR) using the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). The amount of total RNA was spectrophotometrically determined at 260 and 280 nm. RNA integrity

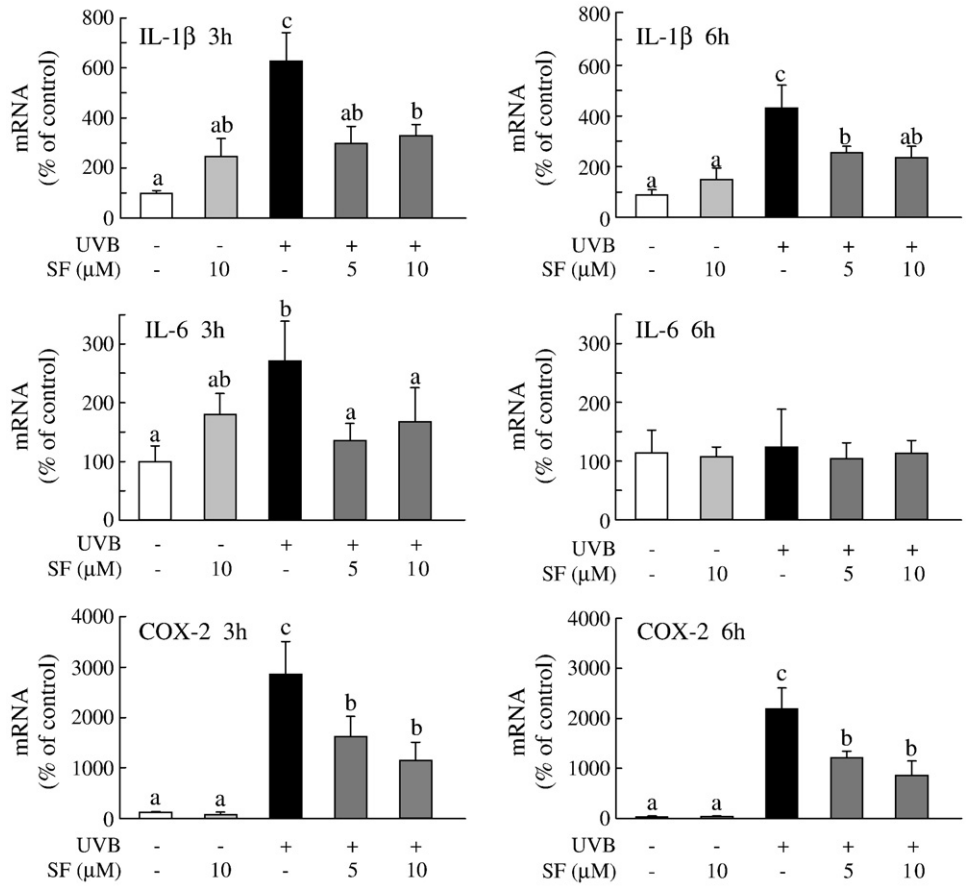

Fig. 4. Effect of SF on UVB-induced mRNA expression of IL-6, IL-1β and COX-2. HaCaT cells ($8–10\times10^5$) were preincubated in the growth medium (DMEM containing 10% FBS) for 24 h. Then, the medium was changed to the test medium (DMEM containing 0.1% FBS supplemented with or without SF), and incubation was performed for 24 h. After being washed with PBS, cells in PBS were exposed to UVB (50 mJ/cm^2). The UVB nontreated cells were also prepared as negative control. These UVB-treated or nontreated cells were cultivated in sample-free test medium for 3 and 6 h. Then, mRNA levels of IL-6, IL-1β and COX-2 were measured by RT-PCR and compared with UVB (−)- and SF (−)-treated cells for 3 h. Values are mean ±S.D. (n=6). Means without a common letter differ; P<05.

was confirmed by visualizing intact 28S and 18S ribosomal RNAs on formaldehyde denaturing agarose gel. From the RNA, cDNA was synthesized using a Ready-To-Go T-Primed First-Strand Kit (Amersham Pharmacia Biotech, NJ, USA). Then, the cDNA was subjected to PCR amplification using a SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) and gene specific primers for IL-6, IL-1β, cyclooxgenase-2 (COX-2) and β-actin (Table 1). PCR condition was 95°C for 1 min, 95°C for 5 s and 68°C for 30 s over 40 cycles. Melt curve analysis was performed following each reaction to confirm the presence of only a single reaction product. In addition, representative PCR products were electrophoresed on a 2.0% agarose gel to verify that only a single band was present. The ratio between the β-actin content in control and test samples was defined as the normalization factor.

2.8. Western blot analysis

The UVB-treated cells or nontreated cells were cultivated in sample-free test medium for 0.5, 6 and 24 h. From the cells, cellular proteins were prepared as previously described [26], and the cellular proteins (40 μg/well) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–20% e-PAGEL, Atto, Tokyo, Japan). The protein bands were then transferred to polyvinylidine fluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After being blocked of nonspecific sites, the membrane was probed with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA). The detection of the antibody reactions was performed with ECL Plus Western blotting reagents (Amersham Pharmacia Biotech). The antibodies used were anti-phospho JNK/SAPK, anti-JNK/SAPK, anti-phospho p38, anti-p38, anti-phospho ERK, anti-ERK (BD Transduction Laboratories, San Diego, CA, USA), anti-human COX-2 (IBL, Takasaki, Japan), anti-mouse COX-2 (Cayman Chemical, Ann Arbor, MI, USA) and anti-β-actin (Cell Signaling Technology).

2.9. Animal study

The mice were divided into four groups (Group 1 was left untreated; Group 2 was UVB irradiated but with no SF treatment; Groups 3 and 4 were fed with SF 1 and 2.5 mg/day, respectively, and were irradiated with UVB). Each mouse orally received SF (dissolved in 100 μl of PBS) or PBS alone once a day for a total of 14 successive days. On Days 9, 11 and 13, mouse skin was directly exposed to UVB irradiation from the vertical position to its back at a dose of 200 mJ/cm^2 at a distance of 22 cm using an EL lamp [8 W, UVL-28, 302/365 nm, 100 V (UVP); 12–13 min of exposure time]. During the feeding period, the mice were allowed free access to laboratory rodent chow and water, and skin thickness was measured daily at three midline sites using a caliper (Digimatic Micrometer 543; Mitutoyo Co., Tokyo, Japan). On Day 14, after 24 h from last UVB exposure, mice were euthanized and skins were collected for Western blotting and histological analysis.

2.10. Skin analysis

Skin samples (about 50 mg) were ground under liquid nitrogen, and then protein was extracted using the same procedure as described above. The extracted protein was analyzed by Western blot analysis. For determination of hyperplasia, the skin was fixed in 4% formalin and embedded in paraffin. Vertical sections (5 μm) were cut, mounted on a glass slide and stained with hematoxylin and eosin [27].

2.11. Statistical analysis

The data were expressed as mean±S.D. We performed statistical analysis using a one-way ANOVA, followed by Scheffe's *F* test for multiple comparisons among several groups. Differences were considered significant at $P<.05$.

3. Results

3.1. In vitro study of skin inflammation inhibition by SF or GRP

To evaluate the role of SF in skin inflammation, the effect on proliferation of HaCaT keratinocytes was tested first under non-UVB irradiation condition. As results, SF promoted HaCaT proliferation when its concentration was under 10 μM, but it inhibited the proliferation above 25 μM (Fig. 2).

Under UVB irradiation condition, UVB caused marked inflammatory responses (i.e., decrease of cell viability and increase of production of an inflammatory marker IL-6) (Fig. 3). SF (10 μM) treatment recovered the cell proliferation to almost basal (non-UVB exposure) levels and also attenuated IL-6 production. SF at high dose (25 μM) showed lower values for both proliferation and IL-6, probably due to its cytotoxic effect as observed in Fig. 2.

3.2. Cutaneous anti-inflammatory mechanism of SF

In the next series of investigations, we evaluated the mechanism by which SF suppresses UVB-induced inflammatory responses by measuring mRNA expression of IL-6 as well as other mediators such as IL-1β and COX-2 in HaCaT. The result of the RT-PCR showed that, although SF (10 μM) did not change these mRNA levels in non-UVB-irradiated cells, it suppressed UVB-induced inflammatory gene expressions, except that of IL-6 at 6 h of incubation (Fig. 4). According to Fig. 4, because SF seems to have more effect on the expression of COX-2, we further focused on COX-2 and Western blot analysis confirmed that SF reduced UVB-induced COX-2 protein expression (Fig. 5A). This effect was supported by the finding that PGE_2 released from HaCaT was dose-dependently suppressed by SF (Fig. 5B). At this time, SF did not affect the basal (non-UVB exposure) level of PGE_2 or COX-2. On the other hand, mitogen-activated protein kinases (MAPK) are implicated in the inflammatory signaling, which facilitates the production of inflammatory mediators [28]. We therefore studied the effect of SF on UVB-induced phosphorylation of MAPK (p38, ERK and

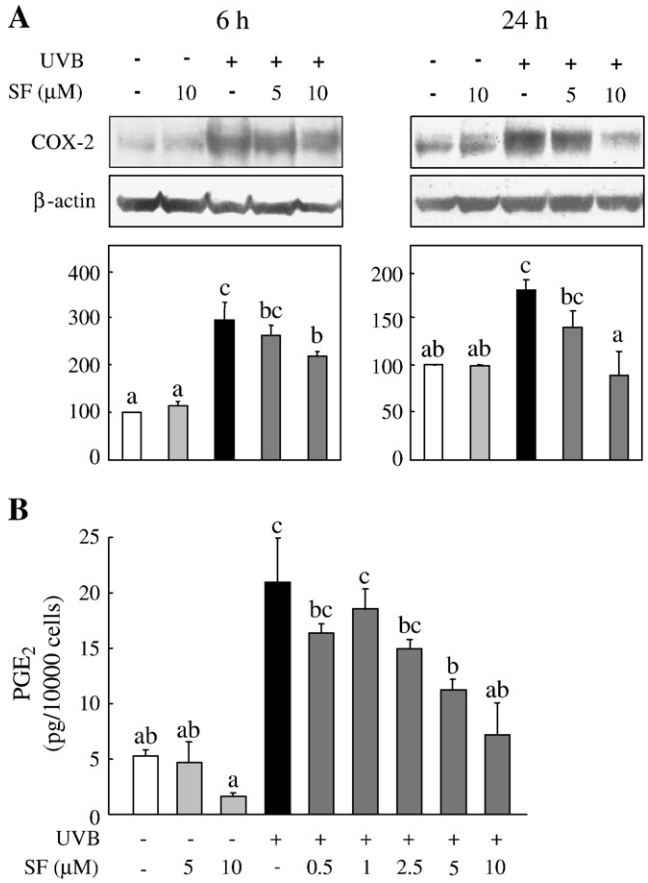

Fig. 5. Effect of SF on UVB-induced COX-2 protein expression (A) and PGE_2 secretion (B). HaCaT cells ($4–8\times10^6$) were preincubated in the growth medium (DMEM containing 10% FBS) for 24 h. Then, the medium was changed to the test medium (DMEM containing 0.1% FBS supplemented with or without SF), and incubation was performed for 24 h. After being washed with PBS, cells in PBS were exposed to UVB (50 mJ/cm^2). The UVB nontreated cells were also prepared as negative control. These UVB-treated or nontreated cells were cultivated in sample-free test medium for 6 and 24 h for Western blot analysis of COX-2 (A). Each Western blot is a representative example of data from three replicate experiments. On the other hand, for analysis of PGE_2 secretion (B), the UVB-treated or nontreated cells were cultivated in sample-free fresh test medium (DMEM containing 0.1% FBS) for 24 h, and aliquot of the conditioned medium was used to measure the levels of PGE_2 by ELISA. Data is expressed as secretion of PGE_2 pg/10,000 cells. Values are mean±S.D. ($n=3$). Means without a common letter differ; $P<.05$.

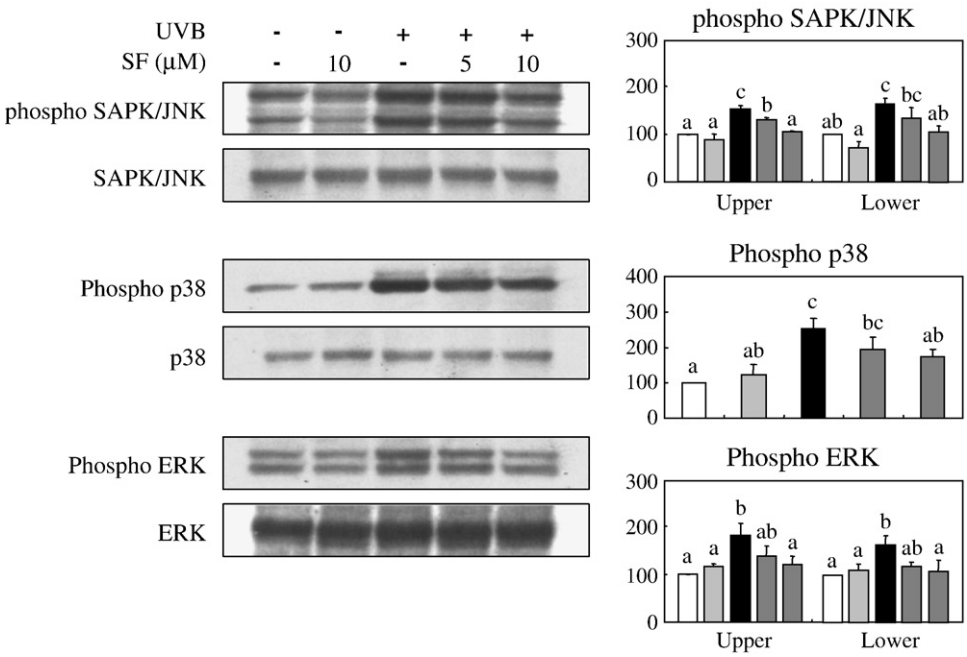

Fig. 6. Effect of SF on UVB-induced expression of intracellular proteins associated with inflammatory reactions. HaCaT cells (4–8×10^6) were preincubated in the growth medium (DMEM containing 10% FBS) for 24 h. Then, the medium was changed to the test medium (DMEM containing 0.1% FBS supplemented with or without SF), and incubation was performed for 24 h. After being washed with PBS, cells in PBS were exposed to UVB (50 mJ/cm^2). The UVB nontreated cells were also prepared as negative control. These UVB-treated or nontreated cells were cultivated in sample-free test medium for 0.5 h for Western blot analysis of p38, ERK and SAPK/JNK. Each Western blot is a representative example of data from three replicate experiments. Values are mean±S.D. (n=3). Means without a common letter differ; P<.05.

SAPK/JNK) by Western blot analysis using phospho-specific antibodies. It was found that SF inhibited all p38, ERK and SAPK/JNK activation without altering total protein levels (Fig. 6), indicating that the inhibition of MAPK activation by SF would attenuate the expression of inflammatory mediators, thereby reducing inflammatory responses in HaCaT.

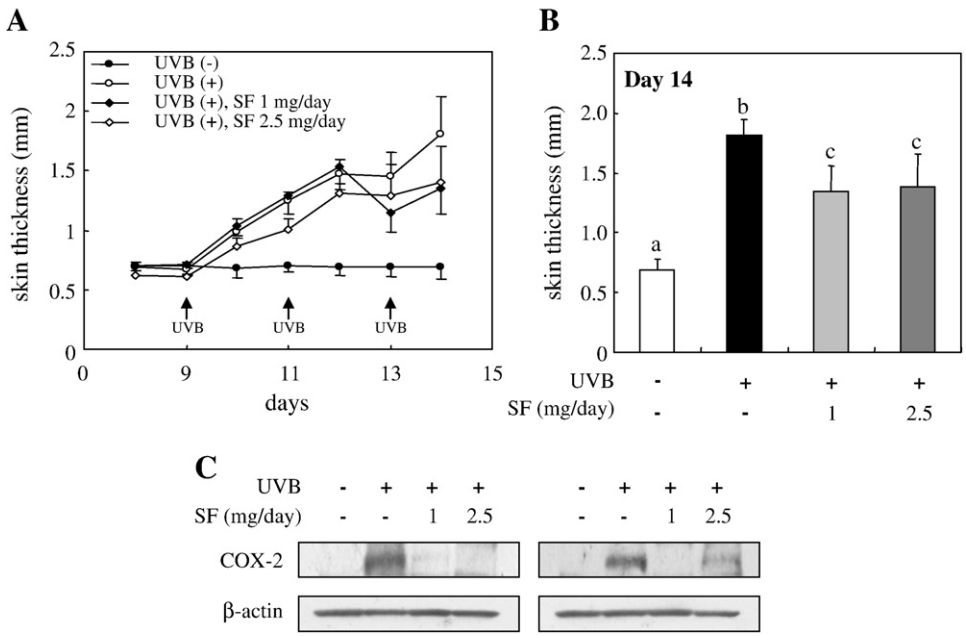

Fig. 7. Effect of oral administration of SF on skin thickening (A, B) and COX-2 protein expression (C) of UVB-irradiated HR-1 hairless mice. The mice were divided into four groups (Group 1 was left untreated; Group 2 was UVB irradiated but with no SF treatment; Groups 3 and 4 were fed with SF 1 and SF 2.5 mg/day, respectively, and were irradiated with UVB). Each mouse orally received SF (dissolved in 100 μl of PBS) or PBS alone once a day for a total of 14 successive days. On Days 9, 11 and 13, mouse skin was directly exposed to UVB irradiation from the vertical position to its back at a dose of 200 mJ/cm^2. During the feeding period, skin thickness was measured daily at three midline sites using a caliper (A, B). Values are mean±S.D. (n=8). Means without a common letter differ; P<.05. On Day 14, after 24 h from last UVB exposure, skins were collected for Western blot analysis of COX-2 (C). Each Western blot is a representative example of data from three replicate experiments.

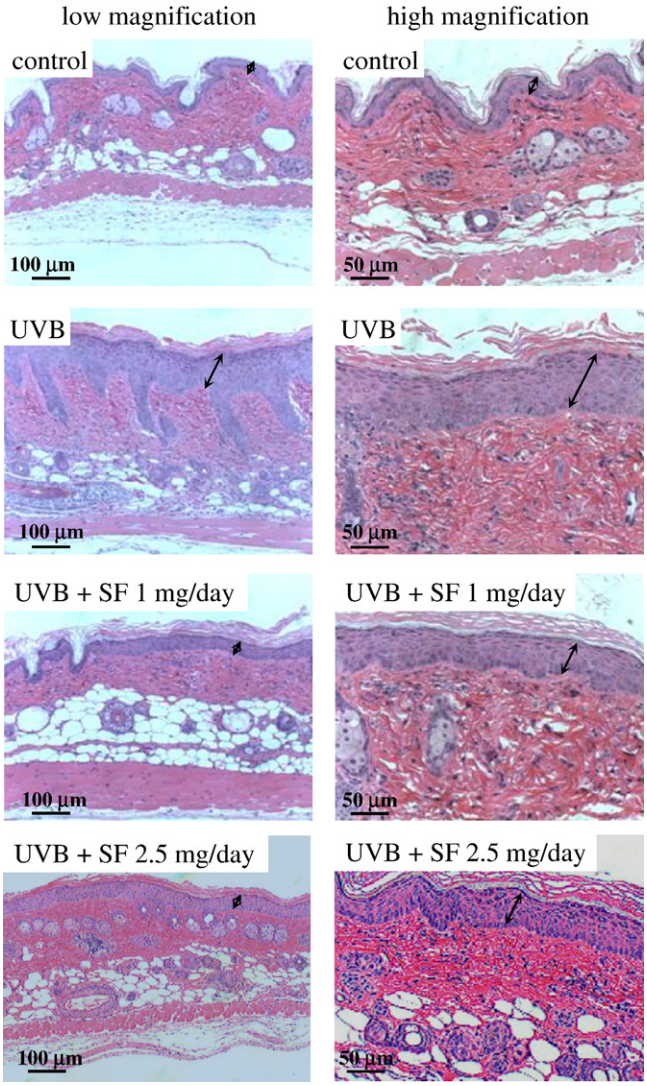

Fig. 8. Effect of oral administration of SF on histology of skin of UVB-irradiated HR-1 hairless mice. Skin samples were fixed in 4% formalin and embedded in paraffin. Vertical sections (5 μm) were cut, mounted on a glass slide and stained with hematoxylin and eosin. The arrow represents epidermal layer thickness.

3.3. In vivo study of skin inflammation inhibition by SF

Finally, we evaluated the *in vivo* inhibitory effect of SF on skin inflammation using HR-1 hairless mice. When the mouse dorsal skin was irradiated with 50–250 mJ/cm^2 of UVB, UVB caused an increase in skin inflammatory markers (skin thickness and COX-2 protein expression) in a dose-dependent manner (data not shown). Based on results, we selected to use 200 mJ/cm^2 of UVB for effectively assessing the effect of SF on these skin inflammatory markers. As shown in Fig. 7, both the UVB-induced skin thickness and the COX-2 protein expression were suppressed by oral administration of SF to the mice. In support of this, histological analysis revealed that, although severe hyperplasia was seen in the skin of UVB-irradiated mice, it was attenuated by SF treatment (Fig. 8). When hyperplasia was evaluated by epidermal thickness, the thicknesses were two to three cell layers for UVB nontreated control mice, eight to nine cell layers for UVB-irradiated mice and three to four cell layers for UVB-irradiated mice receiving SF. In this study, the difference in efficacy between SF dose of 1 and 2.5 mg/day was not observed.

4. Discussion

Exposure of skin to UVB radiation results in a variety of biological effects, including inflammation, sunburn cell formation, immunologic alterations and photo-aging [29–31]. Keratinocytes respond to the major changes in the inflammation and immunomodulation observed after UVB exposure, at least via the UVB-induced release of inflammatory mediators such as cytokines and prostaglandins [32,33]. In this study, we found that treatment of SF with HaCaT keratinocytes suppressed UVB-induced reduction of cell viability and production of inflammatory mediators. These changes would be associated with the ability of SF to suppress MAPK activation. Considering these *in vitro* data as well as *in vivo* findings using HR-1 hairless mice, SF has a potential use against UVB-induced skin damage.

In this study, under non-UVB irradiation condition, HaCaT proliferation was either promoted or suppressed by SF (Fig. 2). Therefore, SF seems to have a biphasic effect such that SF at high concentration (25 μM or more) would cause cytotoxic effect (e.g., apoptosis induction). Under UVB irradiation condition, we found that SF (10 μM) recovered UVB-induced reduction of HaCaT viability as well as suppressed secretion of IL-6 (Fig. 3). This indicates that SF reduces UVB-induced inflammatory responses by suppressing inflammatory cytokines. As for GRP (precursor of SF), it did not affect cell proliferation and secretion of IL-6 (data not shown). A similar tendency was reported for the anticancer effect of SF; that is, SF suppressed the proliferation of tumor cells, while GRP did not [34]. These imply that the isothiocyanate group of SF is necessary to exhibit physiological activity.

UVB radiation causes a temporal change in the cutaneous cytokine micromilieu. Keratinocytes are considered to be major sources of cytokines, chemokines, growth factors and many others [35–37]. The constitutive production of these factors by keratinocytes is rather low, but considerably enhanced by UVB radiation [35–37]. UVB potently induces the release of proinflammatory mediators such as IL-6 and IL-1β from keratinocytes, likely responsible for the onset of the inflammation and the induction of the chemotaxis of the neutrophils and macrophages into the skin [38,39]. Besides these mediators, COX-2 also plays key roles in acute inflammation in UVB-irradiated keratinocytes [40,41]. In this study, as we anticipated, SF (10 μM) suppressed UVB-induced inflammatory gene expressions (IL-6, IL-1β and COX-2) (Fig. 4). In contrast, basal (non-UVB exposure) levels of IL-6, IL-1β and COX-2 were not

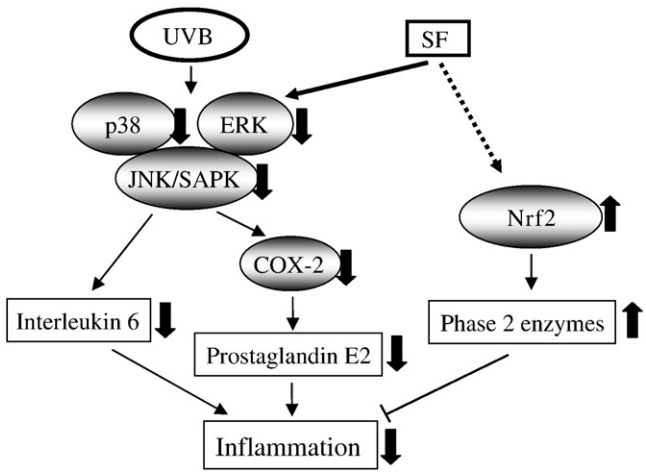

Fig. 9. A proposed mechanism for the anti-inflammatory effects of SF. The effect of SF on Phase II enzymes was speculated from the data of previous studies [6,14].

affected by SF. These findings indicated that SF inhibits UVB-induced inflammatory gene expression, thereby reducing inflammatory responses in HaCaT keratinocytes.

In this study, SF appeared to have an impact on the expression of COX-2 rather than on that of IL-6 and IL-1β (Fig. 4). As mentioned above, COX-2 is one of the most critical enzymes related to inflammation and is required for biosynthesis of PGE_2 from arachidonic acid [42]. PGE_2 is implicated in sunburn reaction and plays a role in skin vasodilatation and erythemal response after solar exposure [43]. Indeed, UVB induces the synthesis of prostaglandins (including PGE_2) in the skin [44]. For these reasons, we focused on COX-2 and PGE_2, and documented that SF inhibits COX-2 protein expression and PGE_2 release from HaCaT induced by UVB (Fig. 5A and B). It is therefore likely that SF acts as an anti-photoinflammatory agent by inhibiting COX-2-dependent PGE_2 production.

Recently, COX-2 expression has been shown to be associated with various intracellular signaling proteins such as p38, ERK and SAPK/JNK [45]. These MAPKs are important upstream regulators of transcription factor activities that control cellular proliferation and differentiation in response to external signals or stimuli [46]. It has been reported that UVB induces COX-2 expression via p38 activation in HaCaT [47] or both p38 and JNK activation in artificial epidermis [48]. ERK is activated by UVB and plays a critical role in transmitting signals [49]. In this study, we observed that p38, ERK and SAPK/JNK were markedly activated in UVB-irradiated HaCaT and that these activations were reduced by SF (Fig. 6). It is therefore possible that SF exerts its inhibitory effect on UVB-induced COX-2 expression by suppressing p38, ERK and SAPK/JNK activities. On the other hand, our present data were still unable to discriminate which kinase is more involved in the regulation of inflammation by SF. Since Woo and Kwon [50] reported that SF suppresses lipopolysaccharide-induced COX-2 expression via inhibition of SAPK/JNK activities, it is likely that SAPK/JNK is an important target for SF. However, some studies reported contradictory results (e.g., up-regulation or no effect on MAPKs) [51,52]. Therefore, further studies are needed and on-going to elucidate the detailed mechanism of action of SF.

To evaluate the *in vivo* effect of SF on UVB-induced skin inflammation, we conducted skin thickening assay using HR-1 hairless mice. Many studies reported the usability of the assay to assess the *in vivo* efficacy of the compound for prevention of photodamage to skin [14,22,53]. As shown in Fig. 7A and B, skin thickening in UVB-irradiated mice was observed, and the thickening was attenuated by oral administration of SF. In addition, SF suppressed UVB-induced COX-2 expression (Fig. 7C) as well as hyperplasia (Fig. 8). Therefore, it is likely that SF acts as an anti-photoinflammatory agent by mainly inhibiting COX-2 expression *in vivo* (Fig. 9). Like SF, inhibition of COX-2 expression has been shown to be an important mechanism of action of some anti-inflammatory compounds, including epigallocatechin gallate [21], resveratrol [22] and curcumin [53].

Recently, Talalay *et al.* [14] have reported that SF-rich extract prepared from broccoli sprouts up-regulated Phase II enzymes in mouse and human cutaneous cells. These authors also found that the extract inhibited UVB-induced inflammation in mice and reduced susceptibility to erythema in human. Therefore, the cutaneous anti-inflammatory effect of SF may be responsible not only for regulation of inflammatory mediators (as demonstrated in this study), but also for suppression of reactive oxygen species via Phase II enzymes induction. This possibility remains to be confirmed, and further studies are needed.

In conclusion, we evaluated the effect of SF on skin inflammation *in vitro* and *in vivo*, and found a potent ability of SF to attenuate UVB-induced skin inflammation by suppressing MAPK activation. Our findings would provide a new insight into the application of SF as well as of cruciferous vegetables for nutraceutical purposes.

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