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Protection against UV-light-induced skin carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts

Albena T. Dinkova-Kostova [a], Stephanie N. Jenkins [a], Jed W. Fahey [a], Lingxiang Ye [a], Scott L. Wehage [a], Karen T. Liby [b], Katherine K. Stephenson [a], Kristina L. Wade [a], Paul Talalay [a,*]

[a] *The Lewis B. and Dorothy Cullman Cancer Chemoprotection Center, Department of Pharmacology and Molecular Sciences, School of Medicine, Johns Hopkins University, 725 N. Wolfe Street, Baltimore, Maryland 21205, USA*
[b] *Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755, USA*

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Abstract

Aerobic life, UV solar radiation, genetic susceptibility, and immune status contribute collectively to the development of human skin cancers. In addition to direct DNA damage, UV radiation promotes the generation of reactive oxygen intermediates that can cause oxidative damage and inflammation, and ultimately lead to tumor formation. Treatment of murine and human keratinocytes with the isothiocyanate sulforaphane elevated phase 2 enzymes and glutathione and protected against oxidant toxicity. Topical application of sulforaphane-containing broccoli sprouts extracts induced the phase 2 response in mouse skin in vivo. Sulforaphane inhibited cytokine-dependent (γ-interferon or lipopolysaccharide) induction of iNOS in RAW 264.7 macrophages. The UV-radiation-induced skin carcinogenesis in 'initiated high-risk mice' was substantially inhibited by broccoli sprout extracts containing sulforaphane. After completion of the UV irradiation schedule (30 mJ/cm^2/session twice a week for 20 weeks), groups of ~30 mice were treated topically on their backs (5 days a week for 11 weeks) with broccoli sprout extract containing either the equivalent to 0.3 μmol (low dose) or 1.0 μmol (high dose) sulforaphane, respectively. At this time point, the tumor incidence had reached 100% in the control mice. Tumor burden, incidence, and multiplicity were reduced by 50% in the animals that received the high dose of protector. Tumor incidence and multiplicity did not differ between the low dose-treated and the control groups, but the low dose treatment resulted in a substantial reduction of the overall tumor burden. Thus, topical application of sulforaphane-containing broccoli sprout extracts is a promising strategy for protecting against skin tumor formation after exposure to UV radiation.

Keywords: Chemoprevention; Phase 2 enzyme; NQO1; Glutathione; Oxidative stress

1. Introduction

Skin cancer incidence is rising steadily and has reached epidemic proportions: the average rise in new skin cancer diagnoses has been 3–8% per year since the 1960s, and nonmelanoma skin cancers are now the most common types of cancer in the United States, with over 1 million new cases per year [1]. Thus, detailed

Abbreviations COX-2, cyclooxygenase 2; GSH, glutathione; γ-IFN, γ-interferon; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NQO1, NAD(P)H-quinone acceptor oxidoreductase 1, also designated quinone reductase.

* Corresponding author.
E-mail address: ptalalay@jhmi.edu (P. Talalay).

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knowledge of the potential risk factors and development of new strategies for prevention are urgently needed.

It is now widely accepted that UV radiation is the main factor responsible for the majority of nonmelanoma skin cancers. In addition to direct DNA damage, the indirect deleterious effects of UV radiation on DNA, proteins, and lipids through the generation of reactive oxygen intermediates and chronic inflammation are processes that can ultimately lead to neoplasia [2]. Early studies have indicated that an antioxidant-supplemented diet (e.g. one containing butylated hydroxytoluene (BHT)) significantly inhibited skin carcinogenesis that was induced either by UV radiation [3] or by polycyclic aromatic hydrocarbons and phorbol ester [4]. Coincidentally, it was shown in our laboratory that BHT and other phenolic antioxidants induce phase 2 detoxification enzymes and protect rodents against the mutagenic metabolites of benzo[*a*]pyrene [5].

The balance between intracellular processes that generate reactive intermediates (e.g. electrophiles, reactive oxygen and nitrogen species) and opposing detoxification and radical scavenging reactions determines the ultimate outcome of exposure to carcinogens [6]. Devising chemical and dietary means to shift the balance towards the latter route, i.e. by induction of enzymes that catalyze phase 2 detoxification reactions, is a major strategy for protection against neoplasia and indeed, a large number of phase 2 inducers have been found to inhibit carcinogenesis in various animal models [7].

The isothiocyanate sulforaphane was isolated as the principal inducer from broccoli [8] guided by the ability to induce phase 2 enzymes. In addition to being one of the most potent naturally occurring phase 2 enzyme inducers known to date, sulforaphane exhibits other biological activities that strongly encourage its development as an anticancer agent, e.g. it stimulates apoptosis and inhibits proliferation [9], is anti-inflammatory [10], and inhibits histone deacetylase [11]. Thus, sulforaphane protects retinal pigment epithelial cells against the toxicity of various biological oxidants, e.g. 4-hydroxynonenal, peroxynitrite, menadione, tert-butyl hydroperoxide [12] as well as against photooxidation generated by all-*trans*-retinaldehyde and UVA light [13]. The degree of protection observed in these experiments was quantitatively correlated with the induction of the phase 2 response. Recently, we demonstrated a linear correlation spanning 6 orders of magnitude of potencies between induction of the phase 2 enzyme NQO1 and inhibition of inflammatory responses (iNOS and COX-2 upregulation by γ-interferon) among a large series of synthetic triterpenoids [14]. Both activities are largely dependent on Nrf2, the principal transcription factor for phase 2 genes. This finding indicates that phase 2 enzyme induction and antiinflammatory activity could contribute collectively to the protective effects of known phase 2 enzyme inducers.

The demonstration that sulforaphane protects laboratory animals against chemical carcinogenesis [15–17] provided a proof of the principle that induction of phase 2 enzymes is a valid strategy for protection against cancer at multiple organ sites, and that protection can be achieved by dietary inducers. In the present study, we asked whether sulforaphane could protect against the most ubiquitous complete carcinogen that is present in our environment, UV light. We used the 'high-risk hairless mouse model' originally developed by Conney and his colleagues [18] to test the hypothesis that topical application of myrosinase-treated broccoli sprout extracts as a source of sulforaphane could protect against UV-light-induced skin tumor formation.

2. Materials and methods

2.1. Preparation and standardization of broccoli sprout extracts

Seeds of broccoli (*Brassica oleracea italica*, cv. DeCicco) were surface-disinfected, spread in layers in inclined, perforated trays, misted with filtered water, and illuminated with fluorescent lamps [19]. After 3 days, sprouts were plunged into boiling water and stirred for ~5 min to inactivate myrosinase and extract the glucosinolates. Glucoraphanin, the precursor of sulforaphane, was the predominant glucosinolate as determined by HPLC [20]. Daikon sprout myrosinase was then added for quantitative conversion of glucosinolates to isothiocyanates [19,21]. This preparation was lyophilized, extracted with ethyl acetate, which was evaporated and the residue was dissolved in 80% acetone: 20% water (v/v) to a final concentration of 50 mM isothiocyanate, as determined by the cyclocondensation reaction [22], of which 90% was sulforaphane. Glucosinolates were absent by HPLC [20]. Preparations for treatment were diluted in 80% acetone (v/v) to contain 1.0 μmol/ 100 μl (high dose) and 0.3 μmol/100 μl (low dose). Inducer bioassay [23] was consistent with previous potency values [8].

2.2. Animals

Female SKH-1 hairless mice (4 weeks old) from Charles River (Wilmington, MA) were acclimatized for 2 weeks in a 12-h light/ 12-h dark cycle, 35% humidity, and free access to water and pelleted AIN 76A diet (Harlan TekLad). Experiments were in compliance with the National Institutes of Health Guidelines, and were approved by the Johns Hopkins University Animal Care and Use Committee.

2.3. Single dose treatment of mice with broccoli sprout extract and processing of skin samples

Seven-week-old female SKH-1 hairless mice (five per group) were treated once topically on their backs with either 100 μl broccoli sprout extract (in 80% acetone) containing 1 μmol of sulforaphane or vehicle. The animals were euthanized 24 h later and rectangular segments (2.5 × 5 cm) of their dorsal skins were removed, pulverized in liquid N_2, and 100 mg of the resulting powder was homogenized in 1 ml of 0.25 M sucrose-10 mM Tris–HCl, pH 7.4. Centrifugation at 14,000 × *g* for 20 min at 4 °C yielded clear supernatant fractions, which were analyzed for protein content and enzyme activity.

2.4. UV irradiation of mice and treatment with protector

UV radiation was provided by UV lamps (FS72T12-UVB-HO, National Biological Corporation, Twinsburg, OH) emitting a mixture of UVB (280–320 nm, 65% of total energy) and UVA (320–375 nm, 35% of total energy). The radiant dose was quantified with a UVB Daavlin Flex Control Integrating Dosimeter and further calibrated with an IL-1400 Radiometer (International Light, Newburyport, MA). The animals were irradiated twice weekly, from 6 to 26 weeks of age, with 30 mJ/cm^2/session. One week later, mice were assigned: 29 animals to each of two treatment groups and 33 to the control group. The mice in the two treatment groups received topical applications of either 100 μl of broccoli sprout extract containing 1 μmol (high dose) or 0.3 μmol (low dose) of sulforaphane; the control group received 100 μl of vehicle (80% acetone). The animals were treated 5 days a week for 11 weeks at which time all animals in the control group had at least one tumor. Tumors (defined as lesions > 1 mm in diameter) and body weight were recorded weekly. Tumor volumes were determined by measuring the height, length, and width of each mass that was larger than 1 mm in diameter. The average of the three measurements was used as the diameter and the volume was calculated ($v = 4\pi r^3/3$). All mice were euthanized on the same day and their dorsal skins were dissected using a rectangular template (2.5 × 5 cm) to include the entire treated areas. Skins were stapled to cardboard, photographed, and fixed in ice-cold 10% phosphate-buffered formalin at 4 °C for 24 h.

2.5. Cell cultures

HaCaT human keratinocytes (a gift from G. Tim Bowden, Arizona Cancer Center, Tucson) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS. PE murine keratinocytes (a gift from Stuart H. Yuspa, National Cancer Institute, Bethesda, MD) were cultured in Eagle's minimum essential medium (EMEM) with 8% FBS, treated with Chelex resin (Bio-Rad) to remove Ca^{2+}. RAW 264.7 macrophages were maintained in DMEM with 10% FBS. All cell lines were grown in 5% CO_2 at 37 °C.

2.6. Quinone reductase (NQO1) and glutathione assays

Cells (20,000 per well) were grown for 24 h in 96-well plates, then exposed to serial dilutions of sulforaphane for either 24 h (for glutathione determination) or 48 h (for NQO1 determination), and finally lysed in 0.08% digitonin. An aliquot (25 μl) was used for protein analysis. Activity of NQO1 was determined by the Prochaska test [23]. To measure the intracellular glutathione levels, 25 μl of cell lysate received 50 μl of ice-cold metaphosphoric acid (50 g/L)-2 mM EDTA to precipitate cellular protein. After 10 min at 4 °C, plates were centrifuged at 1500 × *g* for 15 min and 50 μl portions of the resulting supernatant fractions were transferred to a parallel plate. Total glutathione was determined by rate measurements in a recycling assay [24].

2.7. UV irradiation of cells and determination of reactive oxygen intermediates

PE cells (50,000 per well) were grown for 48 h in 24-well plates and exposed to 1 or 5 μM sulforaphane for 24 h. Cells were incubated with 100 μM 2′,7′-dichlorodinitrofluorescein diacetate (Molecular Probes, Eugene, OR) in 500 μl of fresh medium for 30 min. The medium containing the fluorescent probe was removed, cells were washed with DPBS, and exposed to UVA radiation (10 J/cm^2) using UVA lamps (F20T12/BL/HO;

National Biological, Twinsburg, OH). Control cells were kept in the dark. Cells were detached with trypsin, suspended in 2.0 ml of DPBS, and fluorescence intensity was determined at 520 nm (excitation at 485 nm).

2.8. Assay for inhibition of iNOS upregulation

RAW 264.7 macrophages (5×10^5 cells/well) were plated in 96-well plates, incubated with sulforaphane and either 10 ng/ml of γ-IFN or 3 ng/ml of LPS for 24 h. NO was measured as nitrite by the Griess reaction [25].

2.9. Northern and Western blot analyses

RAW 264.7 macrophages (2×10^6 cells/well) were incubated with sulforaphane and either 10 ng/ml of γ-IFN or 3 ng/ml of LPS overnight. For Northern blots, total RNA was isolated with Trizol reagent (Invitrogen), blotted, and probed with iNOS and GAPDH probes radiolabeled with [α-^{32}P]dCTP with random primers [25]. For Western blots, cell lysates were subjected to SDS/PAGE, transferred to a membrane, and probed with iNOS and β-actin antibodies (Santa Cruz Biotechnology).

2.10. Statistical analysis

Tumor incidence was analyzed by Kaplan-Meier survival, followed by stratified log-rank test and a Wilcoxon test, for equality of survivor functions. Multiplicity was evaluated by ANOVA and comparisons were made on all treatments and on individual, paired treatments (*t*-test). Tumor volume was evaluated by ANOVA with treatment time as a nested variable. Calculations were performed with Stata 7.0 or Excel.

3. Results

3.1. Exposure to sulforaphane elevates NQO1 and glutathione and protects against UV-radiation-generated oxidative stress in keratinocytes

When HaCaT human or PE murine keratinocytes were exposed to sulforaphane, the intracellular levels of NQO1 and glutathione were increased in a dose-dependent manner (Fig. 1(A) and (B)). Especially striking was the magnitude of NQO1 induction (>10-fold) in PE cells without any apparent evidence of cytotoxicity. This differential response could not be fully ascribed to differences in basal levels of NQO1 that were only 20% lower in PE cells compared to HaCaT cells.

We next examined the ability of the sulforaphane-induced phase 2 response to protect against oxidative stress caused by UVA in cultures of murine keratinocytes. We chose UVA for this study, because its genotoxicity is thought to be due primarily to the generation of reactive oxygen intermediates. Thus, when PE cells were treated with 5 μM sulforaphane for 24 h and then exposed to UVA (10 J/cm^2), there was a substantial ~50% reduction ($P<0.001$) in reactive oxygen intermediates generated by the UV radiation as quantified by the fluorescent probe 2′,7′-dichlorodinitrofluorescein [26] (Fig. 2).

3.2. Topical application of broccoli sprout extracts as a source of sulforaphane elevates NQO1 in mouse skin

The activity of NQO1, a marker enzyme of the phase 2 response, was next evaluated in vivo in SKH-1 hairless mice. The animals (five per group) were treated topically with a 100 μl of standardized myrosinase-

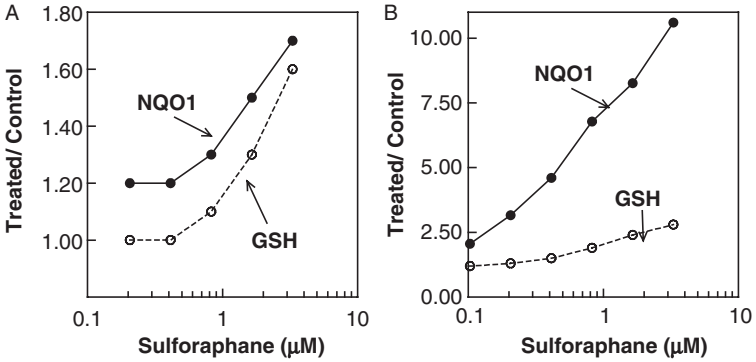

Fig. 1. Induction of NQO1 (●) and elevation of GSH (○) as a function of concentration of sulforaphane in human HaCaT keratinocytes (A) and murine PE keratinocytes (B). Cells (20,000 per well) were plated on 96-well plates and exposed to sulforaphane. GSH and NQO1 levels were measured in cell lysates after 24 and 48 h, respectively. Each data point represents the average of the measurements from 8 different wells. The SD was <5% for all data points.

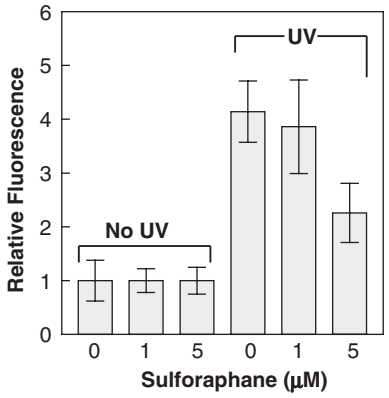

Fig. 2. Protection against UVA radiation-generated reactive oxygen intermediates by sulforaphane in PE murine keratinocytes. Cells (50,000 per well) were plated on 24-well plates, treated with 1 μM or 5 μM sulforaphane for 24 h, washed with DPBS, and then exposed once to UVA (10 J/cm^2). Reactive oxygen intermediates generated by the UV radiation were quantified by the fluorescent probe 2′,7′-dichlorodinitrofluorescein and fluorescence intensity was measured (expressed as a ratio of exposed to unexposed cells).

hydrolyzed broccoli sprout extract containing 1 μmol of sulforaphane. Mice were euthanized 24 h after treatment, their dorsal skin was collected, and the enzyme activity of NQO1 was evaluated in total skin homogenates. There was a substantial ~50% induction ($P <$ 0.001). The specific activity of NQO1 in total skin homogenates of the control (vehicle treated) animals was 5820 ± 303 nmol/min/mg protein, while that of the treated mice was 8590 ± 753 nmol/min/mg protein. We confirmed that the induction of NQO1 by pure sulforaphane in keratinocytes in culture was also observed in vivo in the skin of mice treated with sulforaphane-containing broccoli sprout extract, which was used in the subsequent tumor inhibition experiment.

3.3. Sulforaphane inhibits iNOS upregulation

When RAW 264.7 macrophages were incubated with γ-interferon or lipopolysaccharide together with various concentrations of sulforaphane for 24 h, there was a dose-dependent inhibition of NO formation with an IC_{50} of 0.3 μM for both cytokines (Fig. 3(A)). Northern and Western blot analyses revealed that the synthesis of iNOS mRNA and protein were also inhibited (Fig. 3(B) and (C)). These findings indicate that exposure to sulforaphane suppresses induction of iNOS by either γ-interferon or lipopolysaccharide and attenuates inflammatory responses in macrophages and suggest that sulforaphane-mediated protection against inflammation may also contribute to the inhibition of UV-induced tumor formation in mouse skin in vivo.

3.4. Topical application of broccoli sprout extracts as a source of sulforaphane protects against UV radiation-induced skin carcinogenesis

Exposure of SKH-1 hairless mice to relatively low doses of UVB radiation (30 mJ/cm^2) twice a week for 20 weeks results in 'high-risk mice' that subsequently develop skin tumors in the absence of further UV treatment [18]. This animal model is highly relevant to humans who have been heavily exposed to sunlight as children, but have limited their exposure as adults. It allows the evaluation of potential chemoprotective agents after completion of the irradiation schedule, thus excluding the possibility of a 'light absorbing effect' by

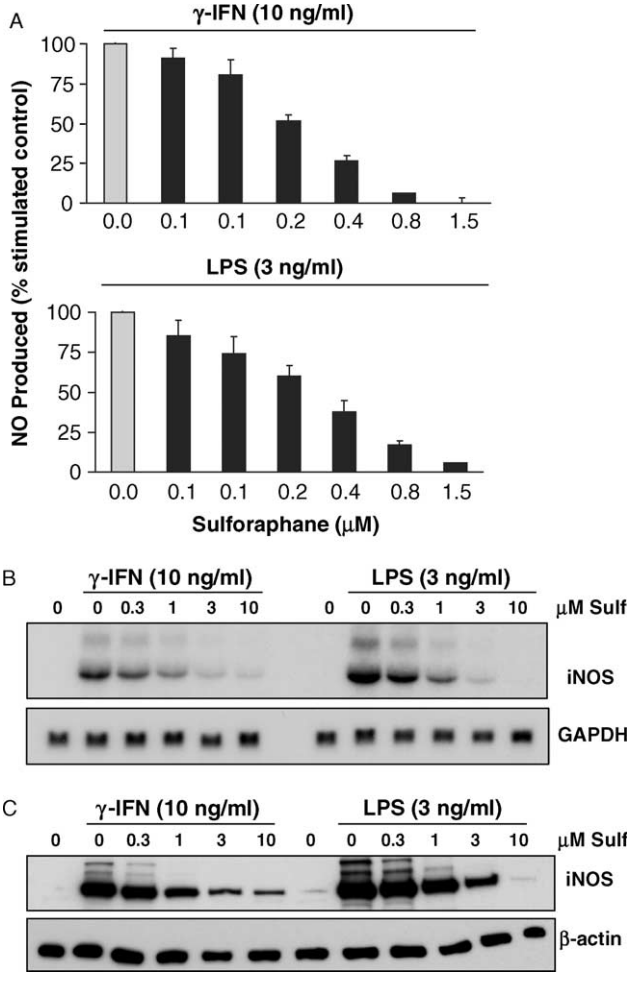

Fig. 3. Sulforaphane inhibits induction of NO formation (A), iNOS mRNA levels (B) and protein levels (C) in RAW 264.7 cells stimulated with γ-interferon or lipopolysaccharide. Cells were treated with various concentrations of sulforaphane and either γ-IFN (10 ng/ml) or lipopolysaccharide (LPS; 3 ng/ml) for 24 h. NO in the medium was measured as nitrite by the Griess reaction (A), and iNOS induction was detected by Northern (B) and Western (C) blotting.

the protective preparations of sprout extracts that are slightly colored. Thus, UV-exposed high-risk mice were treated topically once a day 5 days a week for 11 weeks with 100 µl of standardized myrosinase-hydrolyzed broccoli sprout extracts containing either 0.3 µmol (low dose) or 1 µmol (high dose) of sulforaphane. The control group received vehicle treatment. Body weights and formation of tumors larger than 1 mm in diameter were recorded weekly. There was no difference in average body weight and weight gain among the groups. The body weights (mean ± SD) at the onset of the experiment were: 22.3 ± 1.9 g for the control group, 22.2 ± 1.9 g for the low-dose-treated, and 23.0 ± 1.9 g for the high dose-treated group. At the end of the experiment (31 weeks later), the respective body weights were: 32.1 ± 9.7 g, 31.9 ± 8.8 g, and 32.1 ± 6.9 g. The earliest lesions larger than 1 mm were observed 2 weeks after the end of irradiation, which was 1 week after topical treatment with protector was started. At this time point, three, six and four mice of the control, low dose-treated, and high dose-treated mice, respectively, developed their first tumor.

The high dose-treated animals were substantially protected against the carcinogenic effects of UV radiation. Thus, after 11 weeks of treatment when the experiment was terminated, 100% of the animals in the control group had developed tumors, while 48% of the mice treated daily with sprout extract containing 1 µmol of sulforaphane were tumor-free (Fig. 4(A)). Of note, three animals (two of the control and one of the low-dose-treated groups) were euthanized 1 week before the end of the experiment because they had tumors approaching 2 cm in diameter. Kaplan-Meier survival analysis followed by both a stratified log-rank test and a Wilcoxon test for equality of survivor functions, showed that there was a highly significant difference ($P < 0.0001$) between treatments. The 1-µmol treatment was different from both the 0.3-µmol treatment and the controls, at the 95% confidence level for each of the last three observation periods (weeks 9–11). There was no significant difference between control and 0.3-µmol treatments at any time point.

Similarly, tumor multiplicity was reduced by 58% in the high dose-treated group (Fig. 4(B)): the average number of tumors per mouse was 2.4 for the treated and 5.7 for the control group. The overall effect of treatment on tumor multiplicity was highly significant ($P < 0.001$). ANOVA comparisons of the 1.0-µmol dose level with the control indicated a highly significant overall effect ($P < 0.001$), but differences only became significant after week 9: $P < 0.0794$, 0.0464 and 0.0087 for observations made at weeks 9–11, respectively.

In addition to the reduction in tumor incidence and multiplicity, there was a significant delay of tumor appearance. Whereas 50% of the control animals at risk had tumors at 6.5 weeks after the end of radiation, it took 10.5 weeks for 50% of the high-dose treated animals at risk to develop tumors. Of note, the ability of a protective agent to delay the carcinogenic process is becoming an increasingly attractive concept in chemoprevention.

Although there was no difference in tumor incidence and multiplicity between the low-dose-treated and the vehicle-treated groups (Fig. 4(A) and (B)), the overall tumor burden (expressed as volume in mm^3) per mouse was substantially smaller in the low dose-treated group: 86-, 68-, and 56% smaller at treatment weeks 9, 10, and 11, respectively (Fig. 5). The apparently decreasing effectiveness with respect to treatment time appears to

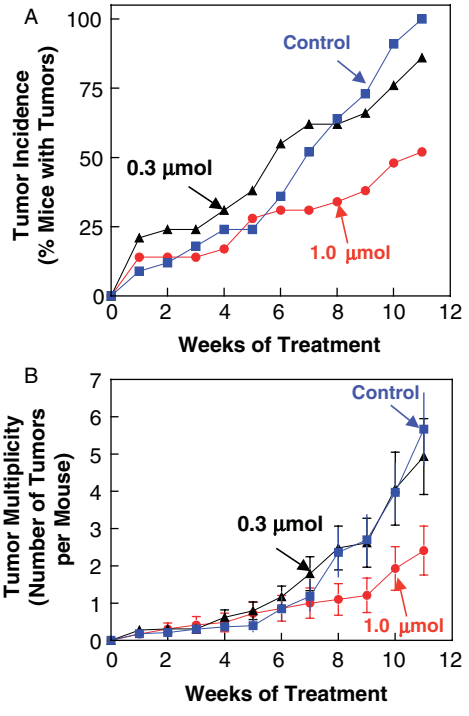

Fig. 4. Inhibition of UVB radiation-induced skin carcinogenesis in high-risk mice by broccoli sprout extracts. SKH-1 hairless mice were irradiated with UVB (30 mJ/cm^2/session) twice a week for 20 weeks and then divided into three groups. The mice from each group were then treated topically with either 100 µl of 80% acetone (control), or 100 µl of sprout extract containing 0.3 µmol sulforaphane (low dose), or 1.0 µmol sulforaphane (high dose) once a day, 5 days a week, for 11 weeks. Average values ± SE are shown.

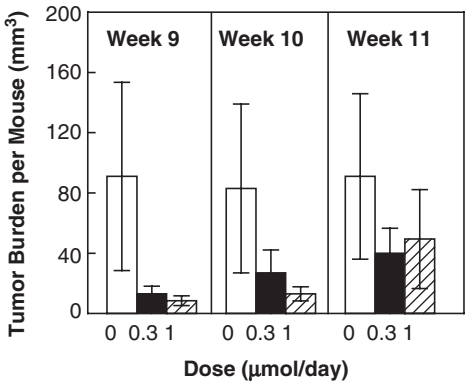

Fig. 5. Inhibition of overall tumor burden in high-risk mice by broccoli sprout extracts. Tumor burden is expressed as total volume of all tumors in mm^3 divided by the number of animals at risk. Average values ± SE are shown. There was a dramatic and highly significant effect ($P < 0.0027$) of concentration (treatment) upon log transformation of tumor volume (ANOVA of concentration using treatment time as a nested variable).

occur because the small tumors ($< 1\ cm^3$) grew rapidly during the last 2 weeks of the experiment. The overall tumor burden in the high dose-treated group was even more dramatically reduced by 91-, 85-, and 46% at treatment weeks 9, 10, and 11, respectively. Interestingly, some of the mice from this treatment group had tumors on the head, where the extract was not applied, but no tumors on their back, where the protective extract was applied.

Although histological characterization of the individual tumors has not been completed, this animal model consistently results in the formation of ~80% small nonmalignant tumors (primarily keratoacanthomas and a few papillomas) and ~20% large malignant tumors (squamous cell carcinoma) [18]. We classified all tumors according to their volumes in two categories: 'small' ($< 1\ cm^3$) (Fig. 6, white bars) and 'large' ($> 1\ cm^3$) (Fig. 6, black bars). Treatment with the sprout extract did not affect the multiplicity of large tumors across the experimental groups: there were 17 large tumors among all 33 animals in the control group, 19 among all 29 animals in the low dose-treated group, and 16 among all 29 animals in the high dose-treated group. In contrast, the broccoli sprout extract produced a dose-dependent inhibition on the number of small tumors: 170, 123, and 54 in the control, low dose-treated, and high dose-treated groups, respectively. It is possible that the unaffected tumors originated from cells that had accumulated mutations caused by direct UV-radiation-induced DNA photoproducts, whereas the extracts inhibited mainly carcinogenic processes resulting from oxidative stress-induced DNA damage. A similar phenomenon has been reported for the soybean isoflavone genistein that inhibited the generation of lipid peroxidation products, H_2O_2, and 8-hydroxy-2′-deoxyguanosine in mouse skin, but had no effect on the pyrimidine dimers formed in response to UV radiation [27].

4. Discussion

UV radiation is the principal factor contributing to nonmelanoma skin cancers in humans. At least four different effects of exposure to UV radiation contribute to the process of carcinogenesis in the skin: (i) direct DNA damage leading to the formation of DNA photoproducts, e.g. cyclobutane-pyrimidine dimers and pyrimidine-pyrimidone products [28]; (ii) oxidative stress-related DNA damage resulting from formation of reactive oxygen intermediates (ROI) [29]; (iii) inflammation [30]; and (iv) immunosuppression that raises tolerance to genetic instability [31].

The protective effect of phase 2 inductions against tumor formation is well established in many model systems. Recently, a new role for the phase 2 enzyme NQO1 has emerged: it was shown to bind and stabilize p53 [32]. Point mutations in *p53* are believed to represent an early event in the development of skin tumors [1,33]. Cells with such mutations can give rise to clones that display genetic instability and, after clonal expansion, ultimately progress to cancers. This observation is in accordance with the finding that *nqo1*-null mice are more susceptible to skin tumor

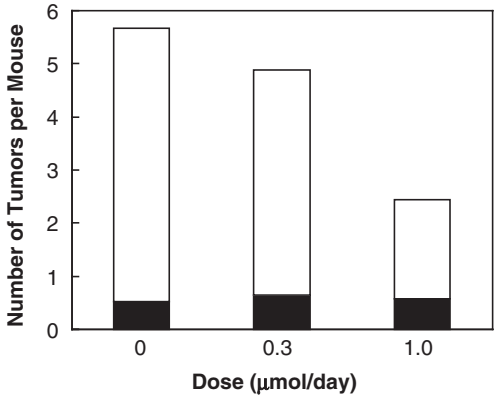

Fig. 6. Number of tumors per mouse (multiplicity) of small ($< 1\ cm^3$, white bars) and large tumors ($> 1\ cm^3$, black bars). Eleven weeks after initiation of treatment with protector (containing 1.0 μmol sulforaphane) or vehicle, the tumor incidence in the control group was 100%, and the experiment was terminated. All mice were euthanized on the same day and the tumor size was measured. Note that the treatment substantially reduced the multiplicity of small, but not of large tumors.

development in a chemical carcinogenesis model and that lack of p53 induction and apoptosis are contributing factors [34]. In humans, there is an inverse association between NQO1-positive genotype and the number of cutaneous basal cell carcinomas [35]. Thus, the elevation of NQO1 in keratinocytes and in mouse skin in vivo upon exposure to sulforaphane could contribute to the protection against the genotoxic effect of UV radiation in at least two ways: (i) by stabilizing p53, and (ii) by acting together with other phase 2 proteins and glutathione to detoxify the UV radiation-generated reactive oxygen intermediates.

GSH can quench radicals directly by hydrogen atom donation, as well as indirectly as a cofactor of the glutathione peroxidase and glutathione transferase systems. Depletion of GSH upon exposure to UV radiation has been observed in the epidermis of both mice [36] and humans [37]. The sulforaphane-induced elevation of GSH indicates that this phenomenon could be also contributing to the observed protection against UV radiation.

In a recent study using dermal fibroblasts growing in culture Hirota et al. (2005) have shown that UVB irradiation at doses higher that 10 mJ/cm^2 causes a decrease in the nuclear levels of Nrf2, the principal transcription factor involved in basal and inducible expression of phase 2 genes [38]. In contrast, UVA irradiation at a dose of 10 J/cm^2 leads to nuclear accumulation of Nrf2, induction of the phase 2 response, and ultimately cellular protection. In agreement with these findings in dermal fibroblasts, our experiments showed that the phase 2 inducer sulforaphane protects keratinocytes against oxidative stress caused by UVA irradiation. We have previously demonstrated that sulforaphane reacts with Keap1, the cellular sensor for phase 2 inducers and repressor of Nrf2 [39]. Exposure to sulforaphane leads to nuclear accumulation of Nrf2 and induction of the phase 2 response [40]. Furthermore, the study by Hirota et al. [38] provided strong genetic evidence that the phase 2 response plays an important role in protection against UV irradiation by demonstrating that dermal fibroblasts from Nrf2 knockout mice are more sensitive to the cytotoxicity of UVA, while cells from Keap1 knockout mice are more resistant.

It was shown nearly 30 years ago that agents considered to be primarily antioxidants, e.g. butylated hydroxytoluene (BHT), significantly inhibited UV-radiation-induced erythema and tumor development in mice [3]. The spectrum of protectors now includes selenium, zinc, difluoromethylornithine, nonsteroidal anti-inflammatory agents, retinoids, as well as plant antioxidants, e.g. silymarin from milk thistle, isoflavones from soybean, perillyl alcohol from citrus fruits, polyphenols from tea, and it has been proposed that their topical application could supplement the use of sunscreens in protecting the skin against UV radiation [see 41–43 for comprehensive reviews]. Green tea, black tea, and their components, e.g. polyphenols, caffeine, and (–)-epigallocatechin gallate, effectively prevent carcinogenesis in UV light-treated high-risk mice when administered either topically or in the diet [18,44]. Green tea polyphenol treatment inhibits UV radiation-evoked erythema and the formation of DNA pyrimidine dimers in human skin [45]. Curiously, (–)-epigallocatechin gallate, much like sulforaphane, exhibits a plethora of biological effects: antioxidant response element (ARE)-mediated induction of phase 2 genes, activation of mitogen-activated protein kinases, stimulation of caspase-3, and apoptosis [46]. Furthermore, pretreatment of human skin with (–)-epigallocatechin gallate prevents UV-induced erythema and associated inflammation, as well as the generation of hydrogen peroxide and nitric oxide, and restores the UV-induced depletion of GSH and GSH peroxidase [37].

Although the exact mechanism(s) by which sulforaphane-containing broccoli sprout extracts protect against UV-light-induced skin carcinogenesis is unknown at present, it is very likely that the observed protection is due to the multiple biological activities of sulforaphane: (i) its indirect antioxidant activity through phase 2 enzyme induction; (ii) its antiproliferative, including cell cycle arrest and apoptosis-inducing activity; and (iii) its anti-inflammatory effects, e.g. inhibition of pro-inflammatory stimuli-induced iNOS and COX-2. It is difficult to establish the relative importance of each in a long-term experiment in which a variety of genetic and epigenetic factors are contributing to the ultimate development of non-synchronized populations of tumors. Nevertheless, we have shown that topical application of sulforaphane-containg broccoli sprout extracts protects against skin tumor formation in a model that is highly relevant to human exposure to UV light.

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