Comparative Medicine
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Vol 61, No 1
February 2011
Pages 39–44

Protective Effect of Inositol Hexaphosphate Against UVB Damage in HaCaT Cells and Skin Carcinogenesis in SKH1 Hairless Mice

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UVB radiation damages keratinocytes, potentially inducing chronic skin damage, cutaneous malignancy, and suppression of the immune system. Naturally occurring agents have been considered for prevention and treatment of various kinds of cancer, including skin cancer. Inositol hexaphosphate (IP6), an antioxidant, is a naturally occurring polyphosphorylated carbohydrate that has shown a strong anticancer activity in several experimental models. We assessed the protective effects of IP6 against UVB irradiation-induced injury and photocarcinogenesis by using HaCaT cells (human immortalized keratinocytes) and SKH1 hairless mice. We found that IP6 counteracts the harmful effects of UVB irradiation and increases the viability and survival of UVB-exposed cells. Treatment with IP6 after UVB irradiation (30 mJ/cm^2) arrested cells in the G_1 and G_2M phases while decreasing the S phase of the cell cycle. Treatment with IP6 also decreased UVB-induced apoptosis and caspase 3 activation. Topical application of IP6 followed by exposure to UVB irradiation in SKH1 hairless mice decreased tumor incidence and multiplicity as compared with control mice. Our results suggest that IP6 protects HaCaT cells from UVB-induced apoptosis and mice from UVB-induced tumors.

Abbreviations: DMBA, 7,12 dimethyl benz(a) anthracene; IP6, inositol hexaphosphate.

Skin cancer is the one of the leading types of cancer in United States, and approximately 1 million cases of nonmelanoma skin cancer occur every year.[30] Human nonmelanoma skin cancer is the most frequently diagnosed malignancy in Caucasians, representing about 30% of all diagnosed cancers worldwide.[13] Both UVA (320 to 400 nm) and UVB (280 to 320 nm) radiation reach the Earth from the sun, but UVB radiation is known to damage keratinocyte DNA by suppressing the immune system and inducing chronic skin damage, cutaneous malignancy, and nonmelanoma skin cancer.[8,18,19,54] Therefore, mechanisms to counteract the DNA damage caused by environmental stress such as UV light are crucial, given that exposure to UVB radiation can lead to massive apoptosis that compromises the natural barrier functions of the skin.[2,36] UVB exposure can cause DNA damage directly or indirectly. Direct damage to DNA leads to the formation of cyclobutane-pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts, whereas indirect DNA damage is due to increases in the level of reactive oxygen species that facilitate DNA oxidation.[8,16,20] Low doses of UVB cause DNA mutation leading to tumor initiation, whereas high doses of UVB lead to apoptotic induction.[32] Apoptosis is a protective mechanism used to remove severely damaged cells that have a risk of becoming malignant. The keratinocytes undergoing apoptosis and eventually cell death are called 'sunburn cells,' and their formation is linked to the severity of UVB-induced DNA damage.[6,7,32] In addition to skin cancer, skin photoaging, and the induction of erythema, there are other adverse effects of UVB radiation. Importantly, these hazardous effects of UVB radiation occur at physiologic doses of UVB intensity at a range of 18 to 30 mJ/cm^2, which corresponds to 1 to 2 min of sunbathing at sea level.[53] With the increased incidence of skin cancer and other damaging effects of UVB exposure, there is a need for novel chemoprevention strategies and interventions. Approaches that involve the use of natural nutritional agents that can prevent UVB-induced damage are promising.

myo-Inositol hexaphosphate (IP6 or phytic acid) is a polyphosphorylated carbohydrate that is ubiquitous in nature in plant and mammalian cells. IP6 is particularly abundant in cereals and legumes.[49] The rapid dephosphorylation of IP6 yields inositol and IP1–5, which are involved in regulating important vital cellular functions.[49,50] Several studies have shown that IP6 can inhibit many types of cancer in different experimental models.[42-45,48-52] IP6 can repair DNA damage, inhibit cell proliferation, and induce differentiation through signal transduction pathways, cell cycle regulatory genes, oncogenes, and tumor suppressor genes.[49,50] Although IP6 has received much attention for its role in cancer prevention and control of experimental tumor growth and progression, the first known and widely accepted role of IP6 is its ability to function as a strong antioxidant. The antioxidant function of IP6 is of great importance because it potentially reduces levels of reactive oxygen molecules, thereby preventing oxygen-mediated carcinogenesis and cell injury.[37,49,50] Because of the anticancer action of IP6,[41-46,48-52] its effect on cell cycle and apoptosis,[15,17,45,46] its strong antioxidant functions,[21,26,37] and its involvement in the repair of DNA damage,[25] we evaluated its potential to protect against UVB radiation-induced cell damage. By using HaCaT

Received: 30 Jun 2010. Revision requested: 22 Aug 2010. Accepted: 04 Oct 2010.
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human immortalized keratinocyte cells, which closely resemble normal human keratinocytes, we studied the in vitro effect of IP6 on UVB-induced apoptosis and survival response.

We previously tested the effect of IP6 on the development of UVB-induced skin tumors in SKH1 mice and demonstrated that IP6 in drinking water decreased tumor incidence by 5-fold and tumor multiplicity by 4-fold.[31] Here, we extended our previous study using the same mouse model but a different mode of IP6 administration and show a photoprotective effect of IP6 against UVB-induced tumor formation even when IP6 is applied topically to SKH1 hairless mice.

Materials and Methods

Cell culture and treatment. The immortalized human keratinocyte cell line HaCaT was obtained from Dr Tim Bowden (University of Arizona, Tucson, AZ) and grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Biofluids Rockville, MD), 1% L-glutamine, and 1% antibiotic (penicillin, streptomycin). Cells were maintained under standard cell culture conditions at 37 °C in a humidified atmosphere of 5% CO_2. IP6 was obtained from Sigma Chemical (St Louis, MO) as the dodecasodium salt. Working solutions (0.05 to 1.0 mM) of IP6 were obtained by diluting a 100-mM stock solution to the required concentrations by using cell culture medium as the diluent.

UVB irradiation protocol. For UVB irradiation, UVB intensities of 15, 30, 60, and 120 mJ/cm^2 were used to expose HaCaT cells to UVB and 1.5 to 7.5 kJ/m^2 were used for mice. Study equipments included UVB lamps (FS40, Westinghouse, National Biological Corporation, Twinsburg, OH) and a UVX digital radiometer with a UVX31 sensor (UV Products, San Gabriel, CA). Approximately 80% of the lamp output was UVB (wavelength 290 to 320 nm), with less than 1% UVC (wavelength less than 290 nm) and 4% UVA (wavelength 320 to 400 nm). Mice received whole-body radiation, and they were not restrained during the procedure. Mice were observed daily for changes in the skin and overall appearance.

Cell cycle progression assay. HaCaT cells were plated in 60-mm tissue culture dishes for 24 h at 37 °C and 5% CO_2. The cells were exposed to 30 mJ/cm^2 UVB irradiation and then treated immediately with 0.5 mM IP6. Cells were harvested 18 h after UVB exposure, and 5 µL RNase (DNase-free) was added to suspensions of 10^6 cells/mL. The cell suspension was incubated at 37 °C for 30 min. The suspension was chilled on ice (2 to 8 °C), and 100 µL propidium iodide was added to the cell suspension. Quantitation of cellular DNA by flow cytometry (Cellular DNA Flow Cytometry Analysis Kit, Roche Diagnostics, Indianapolis, IN) was performed on the same day. Cell distribution was calculated and expressed as mean ± 1 SD for each group of samples.

Apoptosis assay. Apoptotic cell death was assessed by reaction with annexin V and propidium iodide and detected by flow cytometry. This double-labeling assay was used to distinguish between apoptotic, necrotic, and viable cells. HaCaT cells were plated in 60-mm tissue culture dishes for 24 h at 37 °C and 5% CO_2. Cells then were exposed to 30 mJ/cm^2 UVB irradiation, treated immediately with 0.5 mM IP6, and harvested 18 h after the UVB exposure. Aliquots of 1×10^6 cells were washed with PBS (pH 7.4) and centrifuged at $200 \times g$ for 5 min. The pellet was resuspended in 100 µL fluorescently labeled annexin V (Annexin-V-FLOUS Staining Kit, Roche Diagnostics) and incubated for 10 to 15 min at 15 to 25 °C. Samples were analyzed by flow cytometry. Apoptotic, necrotic, and viable cells were calculated and expressed as mean ± 1 SD for each group of samples.

Fluorometric assay for caspase 3. To determine caspase 3 activity, HaCaT cells were plated in 60-mm tissue culture dishes for 24 h at 37 °C and 5% CO_2. The cells were exposed to 30 mJ/cm^2 UVB irradiation and treated immediately afterward with 1.0 mM IP6. Cells were harvested 18 h after UVB exposure for preparation of cell lysates. Aliquots of 1×10^6 HaCaT cells were lysed in hypotonic buffer (25 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol; 2 mM phenylmethylsulfonyl fluoride), 10 µg/mL pepstatin A, and 10 µg/mL leupeptin); 0.10 µL sample was combined with caspase assay buffer comprising 312.5 mM HEPES (pH 7.5), 31.25% sucrose, 0.3125% 3-([3-cholamidopropyl]-dimethyl ammonio)-1 propane sulfonate, 2% DMSO, and 10 mM dithiothreitol containing 50 µM caspase 3 substrate (N-acetyl-DEVD-7-amino-4-methylcoumarin) in white 96-well plates. Negative control wells included 2 µL 2.5 mM specific peptide inhibitor of caspase 3 (N-acetyl-DEVD-CHO). The plate was incubated at 30 °C for 1 h. The free reaction product, 7-amino-4-methylcoumarine, was measured by using a plate reader (Wallac Victor 2 1420 Multilabel Counter, PerkinElmer Life Sciences, Boston, MA) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Caspase 3 activity was reported as relative fluorescent intensity of 7-amino-4-methylcoumarine and expressed as mean ± 1 SD for each group of samples.

Topical treatment and UVB radiation in mice. Female Crl:SKH1-*hr* mice (age, 6 to 8 wk; Charles River Laboratory, Wilmington, MA) were group housed (standard 5 mice/cage) in polysulfone cages with wire mesh covers and fed autoclaved mouse chow and water ad libitum. We used 7087 Teklad soft cob bedding (Harlan, Madison, WI). All mice received AIN76 diet (Harlan Teklad), which is deficient in IP6.[23] All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*[28] and were approved by University of Maryland School of Medicine's Institutional Animal Care and Use Committee. All animals were specified by the supplier to be free of murine viruses, pathogenic bacteria, and endo- and ectoparasites. Mice were divided into 2 groups with 15 mice in vehicle-only group and 8 mice in IP6 topical application group. The IP6 application group received 4% IP6 as a topical cream, and the vehicle group received topical cream deficient in IP6. Cream for topical application with and without IP6 was obtained from Dr Felix Grases; [22] the vehicle cream was composed of almond oil (4%), Thomil ISF (3.8%), stearic acid (1%), lactic acid (1.6%), vitamin F 09929 (2.5%), monestriol GAE (4%), propyl paraben (0.1%), pumol 1618 (4%), controx VP (0.03%), water (72.9%), triethanolamine (0.1%), laurilate S90 (0.3%), glycerin 3699 USP (4.87%), methyl paraben (0.2%), abiol (0.3%), and essence (0.3%). For acclimation, both groups (treatment and control) started receiving topical application with and without IP6 3 d prior to irradiation; we uniformly applied 100 mg of cream on the dorsum of each mouse. On the day of irradiation, cream was applied 1 h prior to irradiation. The UVB flux of the lamps was measured weekly with a UV digital dosimeter. Cages were rotated systematically to compensate for differences in the flux at various positions. The mice were unrestrained as they underwent whole-body radiation. Mice were irradiated 3 times weekly, starting at 1.5 kJ/m^2 with weekly increments of 1.5 kJ/m^2 to a final dose of 7.5 kJ/m^2. All mice were weighed weekly and observed daily for overall appearance and palpable masses.

If erythema was present, we applied topical anesthetics with antibiotics. Tumor formation was monitored for 32 wk.

Statistical analysis. Each experiment was performed at least twice; results were expressed as mean ± 1 SD by using Excel software (Microsoft, Redmond, WA) for calculation. In vitro control and experimental groups were compared by using the Student *t* test (SAS software, SAS Institute, Cary, NC), and the unpaired Student *t* test (SAS software, SAS Institute) was used to assess the difference between control and experimental mice, and differences were considered significant at a *P* value of less than 0.05.

Results

Effects of IP6 on the cell cycle of HaCaT cells exposed to UVB irradiation. We observed a significant ($P < 0.05$) increase in both G_1 and G_2M phases and a significant ($P < 0.05$) decrease in the S phase of cells that were treated with IP6 after being exposed to UVB as compared with cells exposed to UVB radiation only (Figure 1). The average percentage of cells in G_1 phase was 36.11% for the UVB-only group but 67.6% for the IP6+UVB treated group; these percentages were 50% and 7.79%, respectively, for S phase and 13% and 24.6%, respectively, for G_2M phase.

IP6 protects HaCaT cells from UVB-induced apoptosis. The effect of IP6 on apoptosis of HaCaT cells was examined by treating with 0.5 mM IP6 18 h after exposure to 30 mJ/cm^2 UVB irradiation. Cells exposed to UVB radiation without IP6 treatment showed viability of 33.7%, necrosis of 32%, and apoptosis of 32.8% (Figure 2 A). In comparison, in cells treated with IP6 after UVB exposure, viability increased to 62.2%, necrosis decreased to 19.9%, and apoptosis decreased to 9.9%. According to averaged viability, apoptosis, and necrosis from 3 independent experiments (Figure 2 B), treatment of cells exposed to UVB irradiation with 0.5 mM IP6 led to a significant ($P < 0.05$) increase in the percentage of viable cells and a significant ($P < 0.05$) decrease in the percentage of apoptotic and necrotic cells as compared with those in cells exposed to UVB irradiation only.

IP6 inhibited UVB-induced caspase-3 activation in HaCaT cells. The effects of UVB and IP6 on caspase 3 activity in HaCaT cells were assessed by using 1.0 mM IP6 18 h after exposure to 30 mJ/cm^2 UVB irradiation. UVB strongly induced the activity of caspase 3 (Figure 3). However, in the presence of 1.0 mM IP6, UVB-induced caspase 3 activity was significantly ($P < 0.01$) lower than that in cells exposed to UVB irradiation but not treated with IP6 (Figure 3).

Topical IP6 decreased tumor incidence and multiplicity in SHK1 hairless mice. The effect of IP6 on tumor incidence and tumor multiplicity in SHK1 hairless mice was determined by topical application of 4% IP6 in mice after their exposure to increasing doses of UVB radiation. Cumulative results demonstrated that topical application of an IP6-containing cream led to a significant ($P < 0.05$) decrease in cumulative tumor incidence (the proportion of mice bearing at least 1 tumor; Figure 4 A). In addition, IP6 led to a decrease in cumulative tumor multiplicity (average number of tumors per group) as compared with that seen with the vehicle alone. At week 32, the cumulative tumor incidence was 57% in the mice treated with IP6 compared with 71% in the vehicle group ($P < 0.05$; Figure 4 B), and cumulative tumor multiplicity was 86 in IP6 mice but 129 in vehicle mice.

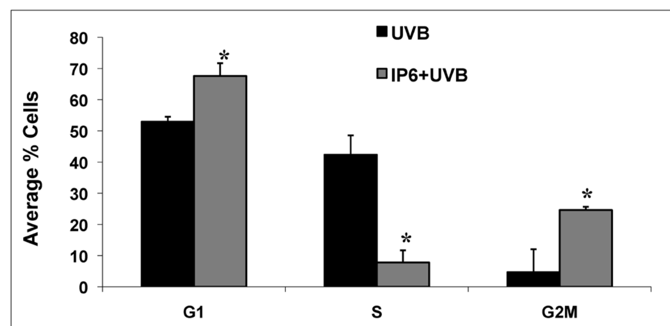

Figure 1. Effect of IP6 and UVB irradiation on cell cycle distribution of HaCaT cells. HaCaT cells were exposed to 30 mJ/cm^2 UVB irradiation and then treated with 0.5 mM IP6. The control HaCaT cells were exposed to UVB and did not receive the IP6 treatment. At 18 h after exposure, cells were harvested and stained with propidium iodide and analyzed by flow cytometry for DNA content. The numbers of cells in G_1, S, and G_2M phases are represented as percentages. Each data point represents the mean ± 1 SD from independent experiments. *, Significantly ($P < 0.05$) different from value for cells exposed to UVB but not treated with IP6.

Discussion

UVB is a complete carcinogen because of its ability to function as an initiator, promotor, and progression factor in the processes involved in skin cancer in the absence of additional initiators or promoters.[4,7] Currently between 2 to 3 million cases of nonmelanoma skin cancer occur globally each year, according to the World Health Organization. Several studies reported that the protective effects of naturally occurring agents with strong antioxidant activity for use against skin carcinogenesis and other biological effects caused by UVB exposure.[1,12,14,27,39] However, the effects of IP6 in prevention of UVB-induced cell injury have not been investigated.

The aim of this study was to determine whether IP6 is effective in protecting human keratinocytes, HaCaT cells, from UVB-induced cell death. A second goal was to establish the effect of topically applied IP6 on tumor incidence and multiplicity in SKH1 hairless mice exposed to UVB radiation. IP6 increased the viability of HaCaT cells exposed to UVB and simultaneously decreased excessive UVB-induced apoptosis and necrosis. We chose HaCaT cells because they are stable, nontumorigenic keratinocytic cells with largely preserved differentiation capacity. HaCaT cells are not malignant when injected into nude mice[35] and are the closest model to normal human keratinocytes.[3] These unique properties make HaCaT cells an excellent model for the study of the skin cancers. HaCaT cells are used extensively as an in vitro model of epidermal skin to investigate the effects of UVB. Other natural compounds similar to IP6, such as silibinin and ketoprofen, have protected HaCaT cells from UVB-induced apoptosis at lower doses of UVB.[11,34]

Hazardous effects of UVB radiation occur at physiologic doses of UVB at an intensity of 18 to 30 mJ/cm^2, a dose corresponding to 1 to 2 min of sunbathing at sea level.[53] Therefore, the doses used in the current study are relevant to human health. Cells with moderately damaged DNA normally are arrested in various stages of the cell cycle so that the damage can be repaired before cells progress to DNA replication or mitosis.[11,33,36] Our results indicated that treatment of HaCaT cells with 0.5 mM IP6 after UVB exposure led to G_1 and G_2M arrest. IP6 is capable of regulating cell

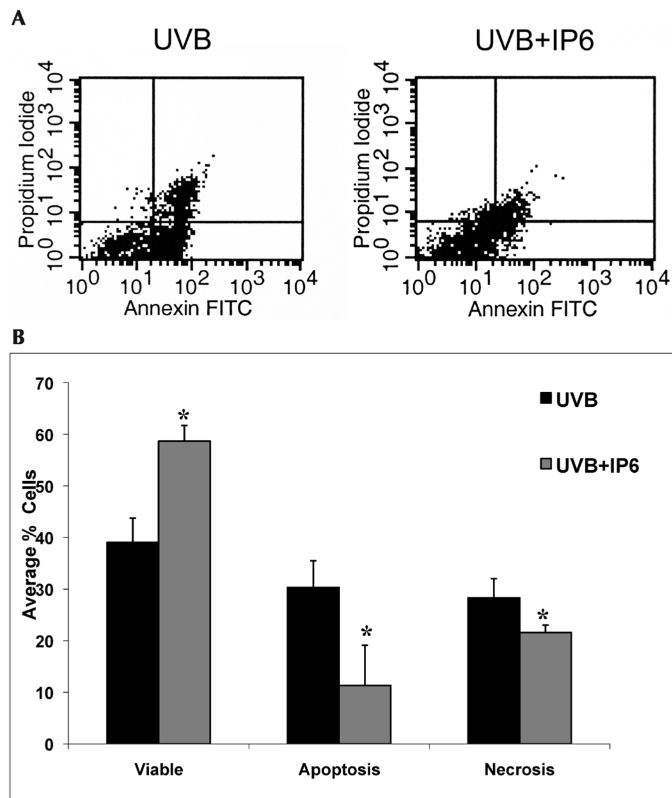

Figure 2. Effect of IP6 and UVB irradiation on viability, apoptosis, and necrosis of HaCaT cells. For annexin V and propidium iodide staining, HaCaT cells were exposed to 30 mJ/cm^2 UVB followed by treatment with 0.5 mM IP6. At 18 h after exposure, cells were harvested, double-stained with FITC–annexin V and propidium iodide, and analyzed by flow cytometry. (A) Fluorimetric distribution plot from a representative experiment. The upper-right quadrant represents necrotic cells, the lower-right quadrant represents apoptotic cells, and the lower-left quadrant represents viable cells. (B) Percentages (mean ± 1 SD) of viable, apoptotic, and necrotic cells from 3 independent experiments. *, Significantly ($P < 0.05$) different from value for cells exposed to UVB but not treated with IP6.

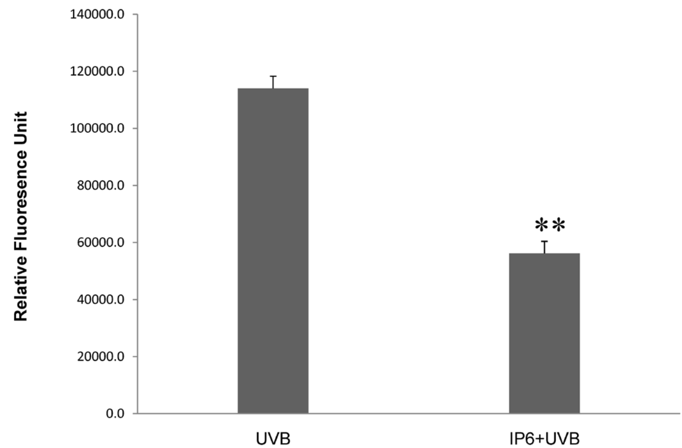

Figure 3. Effect of IP6 and UVB irradiation on caspase 3 activation of HaCaT cells. To assess caspase 3 activity, cells were either not exposed or exposed to 30 mJ/cm^2 UVB irradiation and then either treated or not treated with 1.0 mM IP6. At 18 h after exposure, cells were harvested and a fluorometric assay for caspase 3 was performed. Caspase 3 activity is represented as relative fluorescence units. Each data point represents the mean ± 1 SD from independent experiments. *, Significantly ($P < 0.01$) different from value for the group exposed to UVB but not treated with IP6.

cycle progression by blocking uncontrolled cell division and forcing malignant cells either to differentiate or undergo apoptosis, as occurs in breast, colon,[15] and prostate[46] cancer cells. IP6 treatment of MCF7 and MDA-MB-231 human breast cancer cells and HT29 human colon cancer cells leads to a significant decrease in the proportion of cells in S phase of the cell cycle. Although MCF7 and HT29 cells accumulated in G_0/G_1 phase, MDA-MB-231 cells accumulated only transiently in G_0/G_1 phase. The ability of IP6 to regulate the cell cycle also was demonstrated by decreased expression of proliferation markers, indicating that IP6 disengaged cells from active cycling.[15]

Previous studies have supported the use of HaCaT cells as a suitable model for keratinization studies.[3] Cells of the immortalized human keratinocyte cell line HaCaT are highly sensitive to UVB-induced apoptosis because of their mutant p53 allele,[35] defective NFκB signaling,[5] and upregulation of DNA damage repair proteins.[47] One of the major molecular determinants of apoptosis in the skin is the tumor suppressor gene *p53*, the product of which is involved in numerous cellular functions, such as cell cycle inhibition, regulation of differentiation, transcription and DNA repair. Therefore, cells with mutation in p53 are more susceptible to the tumor-promoting effects of UV radiation and massive apoptosis.[32] The mutant p53 alleles in HaCaT cells suggest that a p53-independent pathway exists for the induction of apoptosis in these cells.[35] In HaCaT cells, IP6 apparently acts in a p53-independent way in affording protection against UVB-caused excessive apoptosis. Other studies using HaCaT cells showed that at UVB intensities of 30 mJ/cm^2 and lower, DNA repair is enhanced.[11] Stress such as DNA damage activates the intrinsic pathway of apoptosis due to disturbances in the mitochondrial membranes, resulting in activation of the initiator caspase 9 and consequently caspase 3.[7,32] Caspases 3, 8, and 9 are involved in UVB-induced apoptosis.[53] We showed that UVB irradiation of HaCaT cells followed by treatment with IP6 leads to a decrease in the levels of activated caspase 3, thus indicating a protective role of IP6 against UVB-induced massive apoptosis. In our experiments, IP6 strongly prevented UVB-induced excessive apoptosis at low irradiation doses, as manifested by decreases in the number of apoptotic cells and in caspase 3 activation, which together indicate a possible survival event in the protective effect of IP6. This outcome suggests that IP6 inhibits the apoptosis of UVB-irradiated HaCaT cells by blocking caspase activity.

The role of IP6 in skin cancer has been investigated previously but not by using HaCaT cells or topical application of IP6 in SKH1 hairless mice. In vitro, IP6 inhibited the growth of melanoma cells.[40] Experiments using 7,12 dimethyl benz(a)anthracene as a carcinogen for the induction of skin cancer showed that IP6 retarded and suppressed tumor growth in a dose-dependent manner.[24] Another previous study using a 2-stage mouse model for skin carcinogenesis showed that IP6 reduces the number of papillomas per animal by 50% when it is given in drinking water during the initiation phase but not the promotion stages of cancer.[29] Mouse skin has been used and accepted widely as an in vivo model to study the effect of UVB-induced photocarcinogenesis and to elucidate the molecular mechanisms of antitumorigenesis.[9,10]

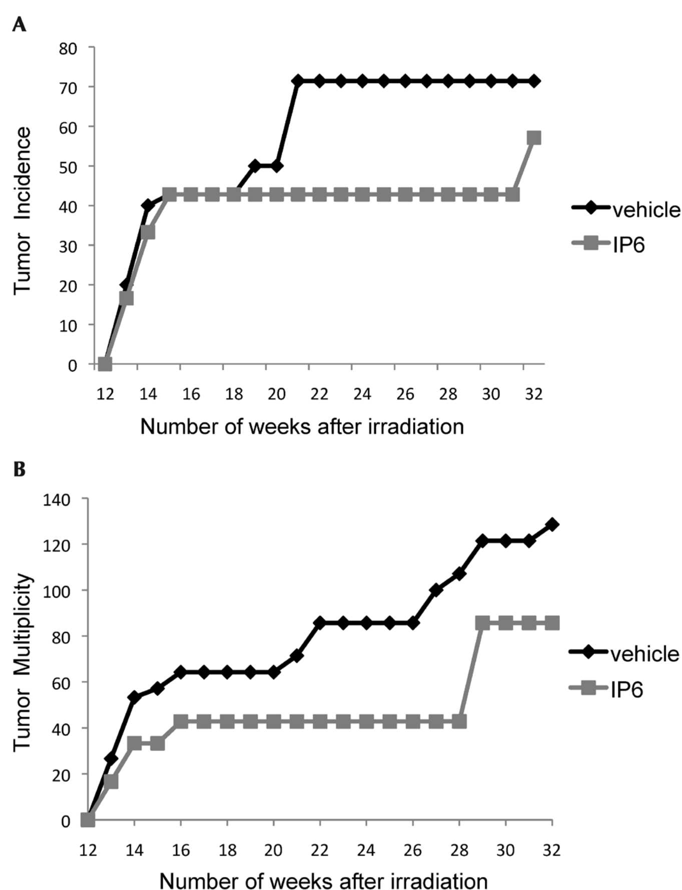

Figure 4. Protective effects of 4% topical IP6 against UVB-induced skin tumors in SKH1 hairless mice. (A) Tumor incidence (proportion of mice bearing at least 1 tumor). By week 32, tumor incidence in the group treated with topical IP6 was 57% compared with 71% in the vehicle group ($P < 0.05$). (B) Tumor multiplicity (average number of tumors per group) was 86 with IP6 compared with 129 in the vehicle group ($P < 0.05$).

Studies using SKH1 mice proved them to be a useful model for UVB-induced skin carcinogenesis.[27] Using SKH1 hairless mice we previously showed that 2% IP6 given in drinking water inhibited UVB-induced nonmelanoma skin tumor formation, as evidenced by significant decreases in tumor incidence and multiplicity.[31] This previous study showed the protective ability of orally administered IP6 against photocarcinogenesis and photoprecarcinogenesis in a SKH1 mouse model.[31] These previous results are consistent with data obtained in the current study, which showed decreases in tumor incidence and multiplicity after topical application of IP6. Similarly, topical application of brown algae polyphenols had antiphotocarcinogenic effects.[27] Because the AIN76 diet is completely IP6-deficient, the only source of IP6 for our mice was the topical cream.[22, 23] Although IP6 is a highly charged molecule, it is easily absorbed through the skin by using either gel or cream.[22] IP6 was absorbed through the skin in a rat model and reached tissues and biologic fluids by crossing the epidermis and dermis to enter the blood stream.[22] The present study demonstrates for the first time that IP6 is effective in protecting HaCaT human keratinocytes from UVB-induced apoptosis and in decreasing tumor incidence and multiplicity through topical application in a mouse model. Future molecular and clinical studies likely should be performed to elucidate the mechanisms underlying the photoprotective effect of IP6.

Acknowledgments

We thank Dr David Zella for providing us with the caspase assay and his laboratory assistant Ms Juahdi Monbo for her technical assistance. Thanks to Mr Doug Allen (Deputy Director) and the technicians of Veterinary Resources (University of Maryland School of Medicine) for all their technical assistance.

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