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Protective effect of topically applied olive oil against photocarcinogenesis following UVB exposure of mice

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Reactive oxygen species have been shown to play a role in ultraviolet light (UV)-induced skin carcinogenesis. Vitamin E and green tea polyphenols reduce experimental skin cancers in mice mainly because of their antioxidant properties. Since olive oil has also been reported to be a potent antioxidant, we examined its effect on UVB-induced skin carcinogenesis in hairless mice. Extra-virgin olive oil was applied topically before or after repeated exposure of mice to UVB. The onset of UVB-induced skin tumors was delayed in mice painted with olive oil compared with UVB control mice. However, with increasing numbers of UVB exposures, differences in the mean number of tumors between UVB control mice and mice pretreated with olive oil before UVB exposure (pre-UVB group) were lost. In contrast, mice that received olive oil after UVB exposure (post-UVB group) showed significantly lower numbers of tumors per mouse than those in the UVB control group throughout the experimental period. The mean number of tumors per mouse in the UVB control, pre-UVB and post-UVB groups was 7.33, 6.69 and 2.64, respectively, in the first experiment, and 8.53, 9.53 and 3.36 in the second experiment. Camellia oil was also applied, using the same experimental protocol, but did not have a suppressive effect. Immunohistochemical analysis of DNA damage in the form of cyclobutane pyrimidine dimers (CPD), (6–4) photoproducts and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in samples taken 30 min after a single exposure of UVB showed no significant difference between UVB-irradiated control mice and the pre-UVB group. In the post-UVB group, there were lower levels of 8-OHdG in epidermal nuclei, but the formation of CPD and (6–4) photoproducts did not differ. Exposure of olive oil to UVB before application abrogated the protective effect on 8-OHdG formation. These results indicate that olive oil topically applied after UVB exposure can effectively reduce UVB-induced murine skin tumors, possibly via its antioxidant effects in reducing DNA damage by reactive oxygen species, and that the effective component may be labile to UVB.

Abbreviations: 6–4PP, (6–4) photoproduct; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; CPD, cyclobutane pyrimidine dimer; NMSC, non-melanoma skin cancer; ROS, reactive oxygen species; SCC, squamous cell carcinoma; UVB, ultraviolet B.

Introduction

Solar ultraviolet radiation (UV), particularly UVB, which has a wavelength of between 290 nm and 320 nm, has been suggested epidemiologically and has been demonstrated experimentally to be the pivotal causal factor for non-melanoma skin cancer (NMSC) in human and in other animals (1). UV has dual actions, which elicit the development of skin cancers: (i) it damages DNA, resulting in mutations of cellular genes crucial for oncogenesis and (ii) induces immunosuppression, which prevents tumor rejection by the host.

Chemical alterations in cellular DNA induced by UV radiation are known collectively as DNA photoproducts. The major types of DNA photoproducts induced by carcinogenic UVB are cyclobutane pyrimidine dimers (CPD) and (6–4) photoproducts (6–4PP). Both types are formed at dipyrimidine sites in DNA. Evidence for the role of photoproduct-induced mutagenicity in human NMSC was provided by studies that showed a high frequency of mutations in the p53 tumor suppressor gene at dipyrimidine sites (2). In contrast to CPD or 6–4PP, which are formed by the direct absorption of UV energy to DNA, an indirect effect of UV on DNA through reactive oxygen species (ROS) is seen in conditions of UV stress. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) is a representative DNA base-modified product generated by ROS (3); however, little is known about the role of 8-OHdG in UV-induced skin carcinogenesis. The formation of 8-OHdG was examined in human skin after UVB irradiation by immunohistochemistry, and increased levels of 8-OHdG were observed in the epidermis after irradiation (4). 8-OHdG induces G-C→T-A transversions during DNA replication. The demonstration of G-C→T-A transversions in the *ras* and p53 genes in UVB-induced skin cancers of mice and in NMSC of humans (5–7) indicates that, in addition to CPD and 6–4PP, 8-OHdG may also play a specific role in UV carcinogenesis.

Recent work has shown that non-DNA chromophores initiate intracellular signaling pathways for gene activation by producing ROS, which alter plasma membrane receptors. For example, ROS can activate AP-1, which plays an important role in tumor promotion (8).

A case–control study in Australia showed a significant inverse relationship between the risk of skin cancer and a high intake of fish, vegetables in general (beans, lentils, peas, carrots, silverbeets or pumpkins), cruciferous vegetables (cabbage, brussel sprouts or broccoli), and β-carotene- and vitamin C-containing foods (9). Some natural agents, particularly plant and vegetable extracts (such as green tea, olive oil, rapeseed oil and safflower oil), have widely been considered to have antioxidant and free radical scavenging capabilities. For example, UV-induced carcinogenesis was suppressed when green tea or green tea polyphenol fractions were topically applied to the mouse skin or were ingested orally in the drinking water (10,11). Epidemiological evidence shows that the incidence of coronary heart disease and certain cancers (such as prostate, breast and colon cancer) is lower in Mediter-

ranean areas where olive oil is the dietary fat of choice (12). However, the effect of olive oil, which is rich in phenolic components (13,14), on UV-induced skin carcinogenesis has not been reported.

Triterpene alcohols from camellia oil have an anti-inflammatory effect on 12-*O*-tetradecanonylphorbol-13-acetate (TPA)-induced inflammation of mouse skin (15). As triterpene derivatives from plants have also been shown to inhibit two-stage chemical carcinogenesis of mouse skin (16), we expected that camellia oil might be protective for photocarcinogenesis.

In this study, we examined the effects of topical application of olive oil and camellia oil on skin tumor formation using hairless mice before or after UVB exposure. Here, for the first time, we report that olive oil topically applied to mice skin after UVB exposure has an anti-cancer effect against murine photocarcinogenesis.

Materials and methods

Animals

Hairless female mice (BALB/cA Kud-hr) aged 6–8 weeks at the beginning of the experiment were purchased from CLEA Japan, Inc. (Tokyo, Japan). They were housed in plastic cages with wire mesh covers, and were fed autoclaved mouse chow and water *ad libitum*. Room illumination was on an automated cycle of 12 h light and 12 h dark, and room temperature was maintained at 22–25°C. All animal experiments were conducted according to the 'Guidelines for Animal Experimentation at Kobe University School of Medicine'.

Olive oil and camellia oil

Extra-virgin olive oil was a gift from DHC Co., Tokyo, Japan. Camellia oil (from *Camellia japonica* Linne) was a gift from Oshima Tsubaki Co., Tokyo, Japan.

UVB-irradiation

Mice were divided into three groups of 15 mice each as follows: group 1: control mice receiving only UVB; group 2: mice painted with olive oil before UVB exposure (pre-UVB group), and group 3: mice painted with olive oil after UVB exposure (post-UVB group). In addition, five mice were sham irradiated with UVB and five mice were painted with olive oil without UVB. Mice were irradiated three times per week at a dose of 3.43 kJ/m^2 each with a bank of six UVB lamps (Torex FL20SE-30/DMR fluorescent sun-lamps with peak emission at 305 nm; Toshiba Medical Supply, Tokyo, Japan). UVB flux was measured by a UVR-305/365D digital radiometer (Opto-Electronic Measuring Instruments, Toshiba Medical Supply, Tokyo, Japan). Immediately before UVB irradiation (group 2) or immediately after irradiation (group 3) ~150 µl of olive oil was painted on to the dorsal skin of the mice using a moist cotton swab. The number of tumors of diameter ⩾1 mm was counted at 0, 5, 10, 15, 18, 20, 24, 25, 27, 28 and 32 weeks of repeated UVB irradiations. Forty-eight hours after the last UV exposure, tumors from each group were randomly biopsied after measuring the size of each tumor. These experiments were repeated twice to confirm the results. The experiment with camellia oil was then done following the same protocol as for olive oil, except that the experiment was not repeated.

Pathological analysis

Biopsied tumors were fixed with formalin and embedded in paraffin, then stained using hematoxylin and eosin. Tumors were classified as papillomas (tumors with papillomatous growth of epidermal cells without atypicality and invasion of tumor cells into the dermis), carcinomas *in situ* (tumors with acanthotic and sometimes papillomatous growth of epidermis with cells of various degrees of atypicality, but with no invasive growth into the dermis) or squamous cell carcinomas (SCC; tumors with atypical cell nests invading the dermis).

Immunohistochemistry

To analyze the effects of olive oil on various types of DNA damage, including CPD, 6–4PP and 8-OHdG, biopsies of normal skin were taken 30 min after single exposure of UVB with or without olive oil application immediately before or after the UVB. Immunofluorescence labeling was then performed to detect photoproducts as follows. Briefly, formalin-fixed, paraffin-embedded sections were deparaffinized, rehydrated and washed in phosphate-buffered saline (PBS). Sections were then incubated in 0.1% trypsin in 0.1 mM Tris–HCl buffer (pH 7.5) for 10 min at room temperature. Nuclear DNA was denatured in 70 mM NaOH in 70% ethanol for 10 min at room temperature. Non-specific binding sites were blocked by 30 min incubation at room temperature in PBS at pH 7.35 containing 10% fetal bovine serum (FBS). Sections were then incubated with mouse monoclonal antibodies to 8-OHdG (antibody N 45.1 (17) at a 1:50 dilution), CPD (antibody TDM-2 (18) at a 1:10 000 dilution) or 6–4PP (antibody 64-M2 (18) at a 1:50 dilution). After incubation overnight at 4°C and three washes in PBS, the sections were incubated for 30 min with biotinylated rabbit anti-mouse link antibody and then for 30 min with streptavidin conjugated to fluorescein isothiocyanate. The sections were then rinsed in PBS, mounted in PermaFluor aqueous mounting medium (Shandon, Pittsburgh, PA) and observed using confocal microscopy.

Quantification was performed using a modification of a previously described method (17). The following equation was used for the quantification of immunohistochemical data:

$$\text{Index} = \Sigma[(X\text{-threshold}) \times \text{area (pixel)}]/\text{total cell number } X > \text{threshold}$$

where X is the staining density indicated by a number between 0 and 256 on the gray scale. Specimens stained with hematoxylin and eosin were used to calculate the number of cells and 8-OHdG, CPD or 6–4PP immunofluorescently labeled specimens were used for densitometric analysis. Color images of 8-OHdG, CPD or 6–4PP immunofluorescently labeled specimens were obtained using confocal microscopy followed by analysis using NIH image freeware (version 1.59). The color image files were opened in gray-scale mode by NIH image. For integrated density determination of immunohistochemical files, a density slice of 100–250 was selected and the 'Measure' command was used. The mean of the integrated density obtained from three independent files was used as a representative value for each group.

Expression of p53 protein was examined using the polyclonal antibody CM-5 (Novocastra, Newcastle upon Tyne, UK) and the streptavidin–biotin–peroxidase staining system (LSAB kit; Dako Carpinteria, CA, USA). Sections of tumor containing >20% p53 protein-positive cells were regarded as p53 positive.

Statistical analysis

Differences in the mean number of tumors per mouse, tumor volumes and 8-OHdG indices of the three independent groups were analyzed by Student's *t*-test. Differences in the histopathological analysis and p53 expression were analyzed by a chi-square test.

Results

Onset, incidence, multiplicity and size of UV-induced skin tumors

The time of onset, when tumors with a diameter of ⩾1 mm initially appeared on the dorsal skin of mice, occurred later in pre- and post-UVB groups than in mice treated only with UVB, in both the first and second experiments (Figure 1A and B and Table I). Data were calculated in terms of tumor incidence (percentage of mice with tumors) and tumor multiplicity (mean number of tumors per mouse). At the end of the first and second experiments, the tumor incidence in the post-UVB group was less than that in the UVB-only and pre-UVB groups (Figure 1A and B). In both experiments, the tumor multiplicity in the post-UVB group was significantly lower than that in control mice treated only with UVB or in mice in the pre-UVB group (Figure 1A and B and Table I). The size of tumors did not differ significantly between the three groups (Table I). Camellia oil did not reduce the tumor incidence and tumor multiplicity although the onset of the tumor was delayed for 2 weeks by topical application before and after UVB exposure (Figure 2, Table II).

Histopathology and p53 protein expression of the tumors with olive oil treatment

Sixty-five randomly selected tumors from mice in the first experiment (23 tumors from mice in the UVB control group, 24 from the pre-UVB group and 18 tumors from the post-UVB group) were examined histopathologically. There were no significant differences in the histopathological characteristics of tumors among these three groups. In all groups, ~10% of

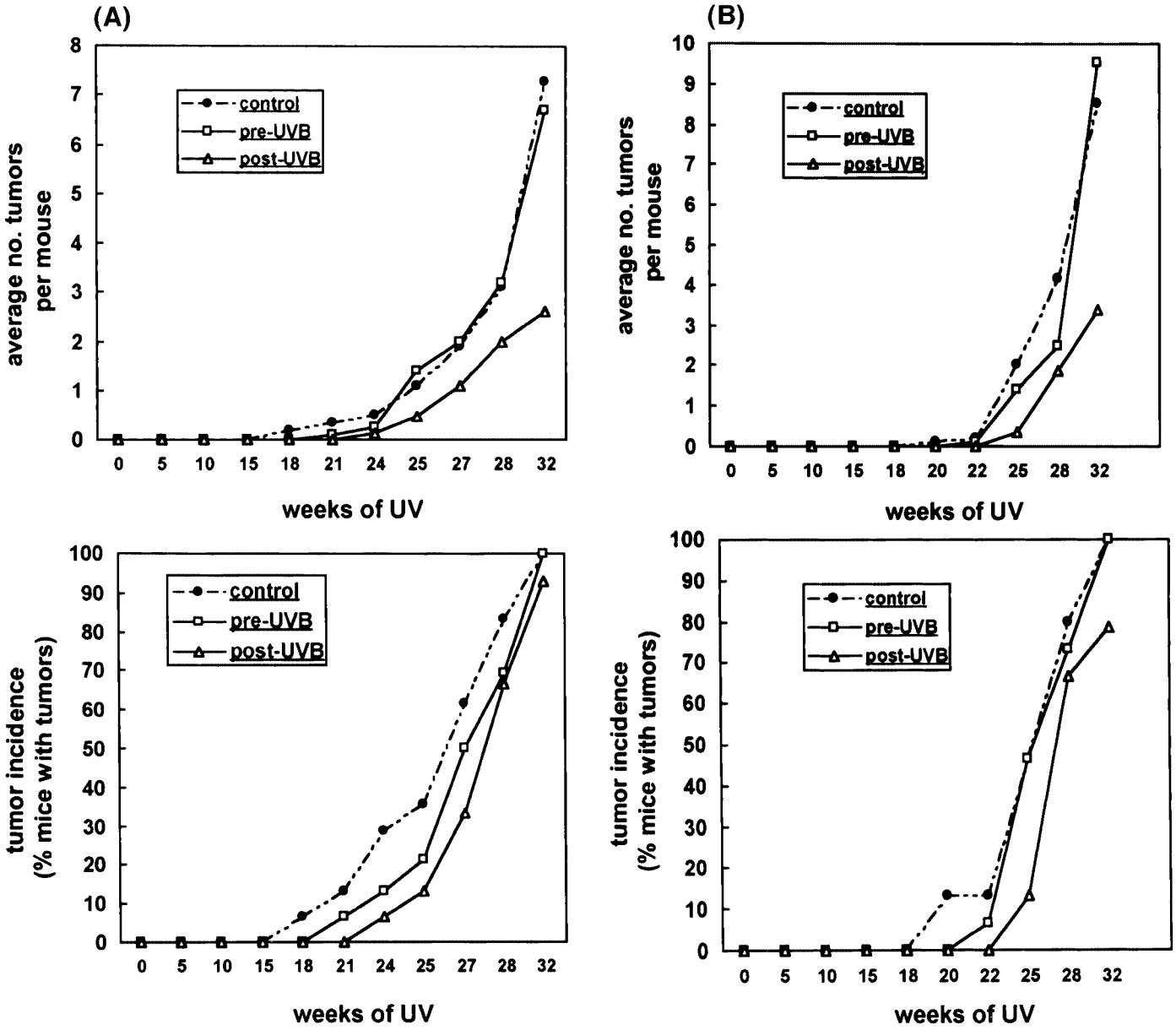

Fig. 1. Effect of topical application of olive oil on UV-induced carcinogenesis of mouse skin. Mice were irradiated with a dose of 3.43 kJ/m^2 three times a week for 32 weeks. Tumors with diameters ⩾1 mm were measured at the times indicated; tumor multiplicity (mean number of tumors per mouse) and tumor incidence (percentage of mice with tumors) were calculated. Mean number of tumors per mouse (top panels) and tumor incidence (bottom panels) in the first (**A**) and second (**B**) experiments.

Table I. Inhibitory effects of topical application of olive oil applied after UVB radiation on UVB-induced mouse skin carcinogenesis

Group[a]	Tumor onset (weeks)[b]		Total number of tumors[c]		Mean number of tumors/mouse[c]		Mean tumor volume[c]	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
1. Control	18	20	88	128	7.33 ± 1.05	8.53 ± 1.21	1.106 ± 0.599	0.10 ± 0.03
2. Pre-UVB[d]	21	22	87	130	6.69 ± 1.72	9.53 ± 1.41	0.052 ± 0.026	0.05 ± 0.01
3. Post-UVB[e]	24	25	37	47	2.64 ± 0.59[f]	3.36 ± 0.82[f]	0.039 ± 0.012[g]	0.06 ± 0.03

[a]There were 15 mice in each group at the beginning of the experiment, but, by the end of experiment 1, there were 12, 14 and 14 in groups 1, 2, and 3, respectively, and by the end of experiment 2 there were 15, 15 and 14 in groups 1, 2 and 3, respectively. Some of the mice died without any sign of skin involvement.
[b]Time at which tumors with a diameter of ⩾1 mm appeared for the first time.
[c]Total number of tumors, mean number of tumors per mouse and mean tumor volume were determined after 95 exposures (32 weeks).
[d]Olive oil was applied immediately before UVB exposure.
[e]Olive oil was applied immediately after UVB exposure.
[f]Statistically different from the control group ($P < 0.001$).
[g]No statistically different from the control group ($P = 0.14$).

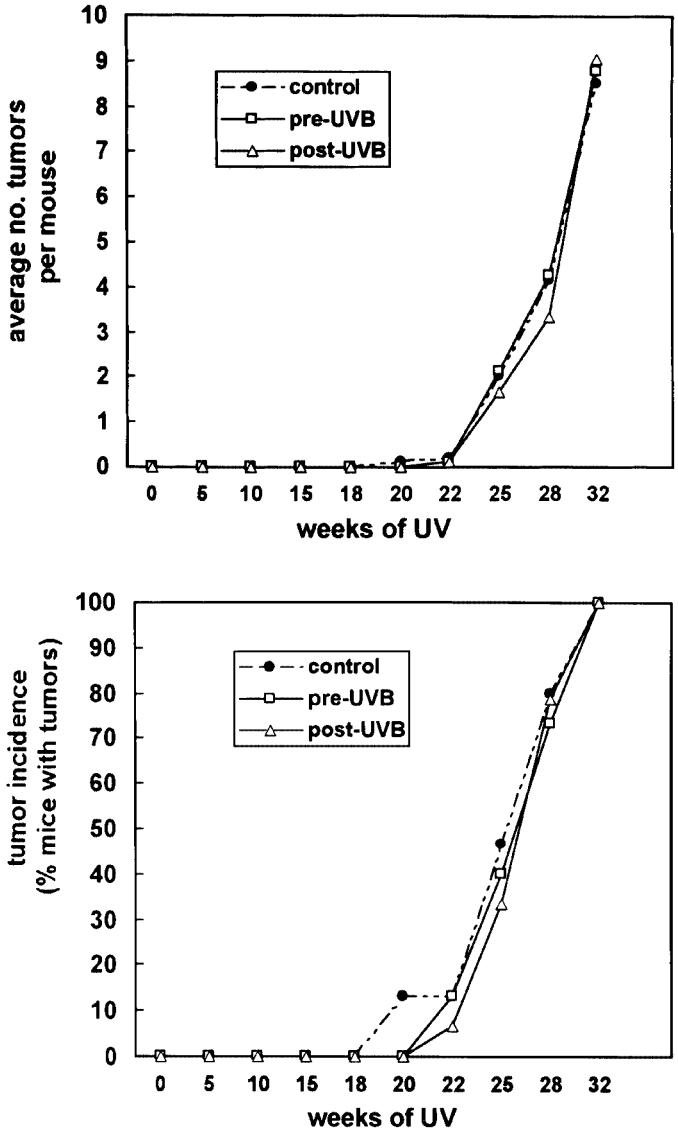

Fig. 2. Effect of topical application of camellia oil on UV-induced carcinogenesis of mouse skin. Mice were irradiated with a dose of 3.43 kJ/m^2 three times a week for 32 weeks. Tumors with diameter ⩾1 mm were measured at the times indicated; the tumor multiplicity/mean number of tumors per mouse (top panel) and tumor incidence/percentage of mice with tumors (bottom panel) are shown.

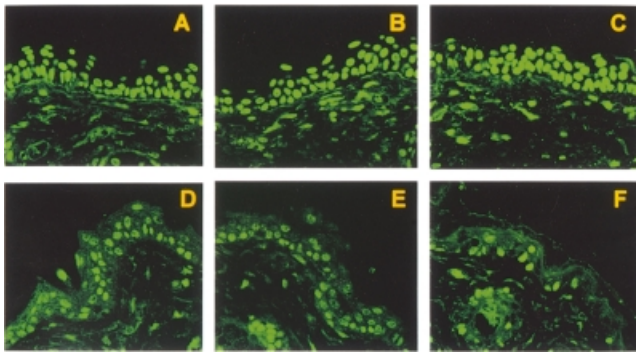

Fig. 3. Effect of topical application of olive oil on UV-induced formation of cyclobutane pyrimidine dimers (CPD) (**A–C**) and 8-OHdG (**D–F**). The dorsal skin of mice was exposed once to UVB at 3.43 kJ/m^2, with or without olive oil application. Biopsies were taken 30 min after exposure and immunofluorescent staining was performed. (A, D) Control UVB only; (B, E) olive oil before UVB (pre-UVB group); (C, F) olive oil after UVB (post-UVB group)

Table II. Effects of topical application of camellia oil on UVB-induced mouse skin carcinogenesis

Group[a]	Tumor onset (weeks)[b]	Total number of tumors[c]	Mean number of tumors per mouse[c]
1. Control UVB only	20	128	8.53 ± 1.21
2. Pre-UVB[d]	22	88	8.80 ± 1.21
3. Post-UVB[e]	22	109	9.08 ± 1.51

[a]There were 15 mice in each group at the beginning of the experiment, but 15, 10 and 12 in groups 1, 2 and 3 respectively, at the end of the experiment. Some of the mice died without any sign of skin involvement.
[b]Time at which tumors with a diameter of ⩾1 mm appeared for the first time.
[c]Total number of tumors and mean number of tumors per mouse were counted after 95 exposures (32 weeks).
[d]Camellia oil was applied immediately before UVB exposure.
[e]Camellia oil was applied immediately after UVB exposure.

tumors were papillomas, 30% were carcinomas *in situ* and 60% were SCC.

There was no significant difference in p53 protein in tumors that developed in the control group or in the two groups treated with olive oil. Of the tumors that developed in control mice and in the pre-UVB group and post-UVB group, 74%, 63% and 56%, respectively, were p53 positive. These rates were not significantly different.

Formation of photoproducts in olive oil-treated skin

After a single exposure of mouse skin to UVB, formation of CPD, 6–4PP and 8-OHdG could be seen in the nuclei of keratinocytes in the epidermis, whereas no photoproduct formation was detected in control non-irradiated skin. No significant difference in photoproduct formation was observed between samples from the UVB-exposed control mice and the pre-UVB group. In contrast, the post-UVB group showed less formation of 8-OHdG in the nuclei of epidermal cells, although the formation of CPD and 6–4PP did not differ from that in the control and pre-UVB groups (Figure 3). When olive oil previously irradiated with UVB (3.43 kJ/m^2) was painted on mouse skin after UVB exposure, the 8-OHdG formation was not reduced to the extent seen in the post-UVB group (data not shown).

The 8-OHdG, CPD and 6–4PP index was determined by measuring the positive staining of nuclei in keratinocytes; there was a significant difference in 8-OHdG index between the UVB control group and the post-UVB group ($P < 0.05$). In contrast, the pre-UVB group and mice treated with UVB-treated oil showed no significant difference in 8-OHdG index compared with the UVB control group (Figure 4). In the indices of other photoproducts (CPD and 6–4PP), there were no significant differences from the control UVB group (data not shown).

Discussion

UVB irradiation modifies DNA both directly, by forming CPD and 6–4PP, and indirectly, via ROS, which produce 8-OHdG. These types of DNA damage induce mutations in genes crucial for cell proliferation and/or apoptosis. Acute UVB exposure also activates transcription factors through signal transduction from the cell surface, possibly initiated by ROS, and these lead to inflammation and tumor promotion. Antioxidants have

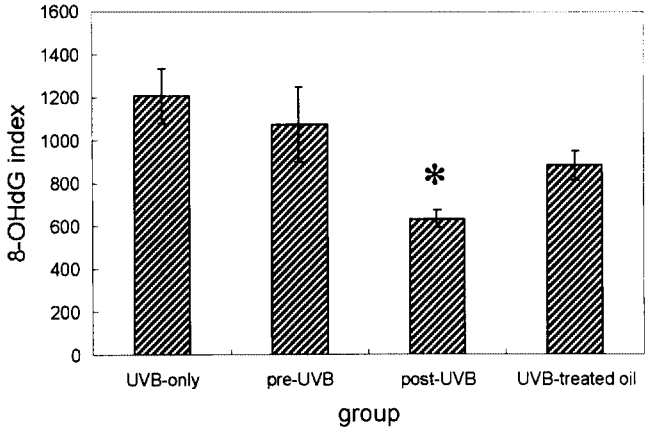

Fig. 4. Olive oil topically applied after UVB radiation reduces 8-OHdG formation. Mice were exposed to UVB with or without olive oil application (immediately before or after UVB). Biopsies of normal skin were taken 30 min after a single exposure to UVB. In addition, UVB-irradiated olive oil was topically applied to mice after a single UVB exposure. Immunofluorescent staining was performed and positive staining was quantified for 8-OHdG index using NIH image software. Data are expressed as the means ± SE from three independent samples. *Statistically different from control UVB group ($P < 0.05$).

been shown to delay the onset of, and to reduce the incidence of, UVB-induced skin tumors experimentally. Taken together, ROS are considered to play specific roles in photocarcinogenesis. In this study, we demonstrate that extra-virgin olive oil topically applied after UVB exposure reduced UVB-induced mouse skin tumors. However, olive oil applied before UVB only delayed the development of UVB-induced mice tumors by 2–3 weeks. Since camellia oil did not prevent photocarcinogenesis, the suppressive effect by olive oil was not due to the general effect of plant or vegetable oils but was possibly limited to the special component of olive oil.

Of the antioxidants, tea polyphenols have been the most extensively studied for their suppressive effects on photocarcinogenesis and both oral and topical administration of tea polyphenols inhibits photocarcinogenesis in mice (10,11,19). The major components of tea polyphenols, which include (–)-epicatechin-3-gallate, (–)-epigallocatechin and (–)-epigallocatechin-3-gallate, have been shown to be strong antioxidants in both hydrophilic and hydrophobic systems. The mechanism(s) of this effect includes the induction of apoptosis, protection against UVB-induced immunosuppression by modulating cytokines, and the scavenging of ROS and blocking of UV-induced oxidative DNA damage, 8-OHdG (20–22).

The ability of olive oil to scavenge ROS has been recently demonstrated and contributes to the low incidence of coronary heart disease and certain types of cancer in the Mediterranean where virgin olive oil is the main source of dietary fat. Phenolic compounds in virgin olive oil, such as hydroxytyrosol and oleuropein, have been shown to act as antioxidants (13,14,23). It has also been shown that hydroxytyrosol in extra virgin olive oil is highly protective against DNA damage caused by peroxynitrite, which is formed by superoxide radicals and nitric oxide (24). There are unusually high concentrations of squalene in olive oil as compared with other common fats and oils consumed by humans (25). Squalene in olive oil is also an antioxidant (26). It inhibits farnesylation of Ras oncoproteins by inhibiting β-hydroxy-β-methylglutaryl-CoA reductase. It inhibits 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis (27) and azoxymethane-induced colonic aberrant crypt foci (28). The anti-tumor promoting activity of squalene in chemical carcinogenesis in mouse skin has also been reported (29). To our knowledge, the present study is the first one that has examined the effect of olive oil on photocarcinogenesis in hairless mice and has demonstrated an inhibitory effect following topical application.

The reduced formation of UV-induced 8-OHdG in epidermal cells in the post-UVB group compared with UVB-only mice and the pre-UVB group indicates that the inhibition of UVB-induced photocarcinogenesis can be attributed to the scavenging activity of olive oil against ROS. The fact that the photocarcinogenesis-suppressing effect was stronger in the post-UVB group than in the pre-UVB group is consistent with the finding that there was less 8-OHdG in the epidermis of the post-UVB group than in the pre-UVB group. These results suggest that olive oil has no sunscreen effect and that its antioxidant effect could deteriorate upon UVB irradiation. The result showing that the application of olive oil previously irradiated by UVB did not reduce 8-OHdG formation compared with unirradiated olive oil confirms the destruction of antioxidant activity in olive oil by UVB. Whether the reduced antioxidant activity of olive oil irradiated by UVB can also be demonstrated by other experimental assays is a matter for future study. The identification of the component in olive oil that is responsible for the suppression of photocarcinogenesis and that is labile to UVB irradiation will also be important. Because it is shown that UV exposure *in vitro* and *in vivo* lipoperoxidates squalene (30), squalene might be the appropriate target for future study. If this was the case, both antioxidant and anti-Ras effects of squalene might have resulted in the prevention of photocarcinogenesis. In contrast to olive oil, the protective effect against photocarcinogenesis was observed by topical application of green tea polyphenols before UVB irradiation (10). However, in that study, green tea was applied several days before UVB treatment and the green tea was not in direct contact with UVB as in our experiments. It also needs to be determined whether polyphenolic compounds in olive oil are labile to UVB.

The fact that camellia oil did not protect against photocarcinogenesis might be a result of it not containing polyphenolic compounds and squalene.

Mutation of the p53 tumor suppressor gene is presumed to be an early event of photocarcinogenesis (31), which may be associated with the initiation step. In this study, the rates of p53 protein over-expression in skin tumors, which frequently indicates a gene mutation, did not differ significantly among the three groups, indicating that olive oil did not affect the quality of the initiation but delayed this step. DNA sequence analyses of the tumors will eventually be necessary to determine whether olive oil affected the quality of gene mutations or not.

It has been reported that oral feeding of tea polyphenols protects against photocarcinogenesis more effectively than topical application (10). The effect of olive oil on photocarcinogenesis when given orally also needs to be determined, therefore. The specific mechanism(s) of protection against photocarcinogenesis, in addition to the antioxidant activity, needs to be studied, because olive oil may induce apoptosis or protect against UVB-induced immunosuppression, as has been demonstrated for tea polyphenols.

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