Inhibitory Effects of Dietary *Spirulina platensis* on UVB-Induced Skin Inflammatory Responses and Carcinogenesis

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Reactive oxygen species produced in response to UVR are important in skin tumor development. We have previously reported that deficiency of the *Ogg1* gene, encoding the repair enzyme for 8-oxo-7,8-dihydroguanine (8-oxoG), increases skin tumor incidence in mice upon repetitive UVB exposure and modulation of UVB-induced inflammatory response. *Spirulina platensis* is used as a human food supplement because it contains abundant nutritional and antioxidant components. Therefore, we investigated the inhibitory effects of *S. platensis* on UVB-induced skin tumor development in *Ogg1* knockout-(KO) mice and the wild-type (WT) counterpart. Dietary *S. platensis* suppressed tumor induction and development in both genotypes compared with our previous data without *S. platensis*. Induction of erythema and ear swelling, one of the hallmarks of UVB-induced inflammatory responses, was suppressed in the skin of *Ogg1*-KO mice and albino hairless mice fed with dietary *S. platensis*. Compared with untreated mice, *S. platensis*-administered mice showed significantly reduced 8-oxoG formation in the skin after UVB exposure. Moreover, we found that *S. platensis* effectively downregulated the signal proteins p38 mitogen-activated protein kinase, stress-activated protein kinase/c-Jun N-terminal kinase, and extracellular signal–regulated kinase after UVB exposure especially in *Ogg1*-KO mice. Our results suggest that *S. platensis* exerts antitumor effects against UVB irradiation in the skin through its anti-inflammatory and antioxidant effects.

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INTRODUCTION

UV light is a carcinogenic component of sunlight and is widely known as one of the most relevant risk factors for skin cancers (Slaper *et al.*, 1996; Urbach, 1997; Howe *et al.*, 2001). Irradiation of mouse skin with UVB (wavelength, 280–320 nm) causes various photochemical reactions, such as induction of DNA damage and the subsequent gene mutation, which may lead to skin cancer development (Nishigori, 2006). Previous studies have demonstrated that UVB induces the formation of 8-oxo-7,8-dihydroguanine (8-oxoG) in mice through UVB-induced reactive oxygen species (ROS; Hattori *et al.*, 1996; Nishigori *et al.*, 2004). The *Ogg1* gene encodes a repair enzyme that removes the oxidized base 8-oxoG-DNA from DNA (Maki and Sekiguchi, 1992; Aburatani *et al.*, 1997; Nakabeppu *et al.*, 2010). In a previous series of studies, *Ogg1*-KO mice showed increasing frequency of mutations but did not exhibit cancer-prone phenotypes in livers and testes (Klungland *et al.*, 1999; Minowa *et al.*, 2000). On the other hand, Sakumi *et al.* demonstrated an increase in the rate of spontaneous lung cancer development in *Ogg1*-KO mice (Sakumi *et al.*, 2003). In our previous study, we found that *Ogg1*-KO mice had a highly tumorigenic phenotype, more numerous skin cancers, and much faster tumor development upon repetitive UVB exposure compared with WT mice (Kunisada *et al.*, 2005).

Spirulina platensis (*S. platensis*) is a blue-green filamentous alga that is currently gaining increasing attention because of its nutritional benefits. Recently, *S. platensis* has been reported to have potential roles in the inhibition of the NADPH oxidase activity (McCarty *et al.*, 2010; Zheng *et al.*, 2013). Owing to its high content of proteins (60–70%) and vitamins (e.g., vitamin B_{12} and provitamin A (β-carotene)), minerals (e.g., iron), and essential fatty acids (e.g., γ-linolenic acid),

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Abbreviations: ERK, extracellular signal–regulated kinase; IL, interleukin; KO, knockout; MEFs, mouse embryonic fibroblasts; PCB, phycocyanobilin; p38 MAPK, p38 mitogen-activated protein kinase; ROS, reactive oxygen species; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; TLR, Toll-like receptor; WT, wild-type; 8-oxoG, 8-oxo-7,8-dihydroguanine

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S. platensis has been produced widely as a food supplement (Belay *et al.*, 1993). Several previous toxicity studies showed that there were no adverse effects in experimental animals after short-term and long-term ingestion of dietary *Spirulina (S. platensis* and *Spirulina maxima*), even after administration of high doses (Salazar *et al.*, 1998; Hutadilok-Towatana *et al.*, 2008). In addition, *S. platensis* has been reported to have various beneficial effects, such as anticancer (Dasgupta *et al.*, 2001; Ismail *et al.*, 2009; Grawish *et al.*, 2010), hepatoprotective (Ismail *et al.*, 2009; Lu *et al.*, 2010), antiviral (Hayashi *et al.*, 1996a, b), anti-allergic (Kim *et al.*, 1998), cardioprotective (Khan *et al.*, 2005), and neuroprotective effects (Bermejo-Bescos *et al.*, 2008). However, the effects of *S. platensis* on UVB-induced responses in the skin still remain unclear. In the present study, we investigated whether dietary *S. platensis* affects skin tumor development in *Ogg1* knockout (KO)-mice induced by long-term UVB exposure and demonstrated its inhibitory effects. Furthermore, we examined whether *S. platensis* suppressed the inflammatory response caused by UVB as we had earlier found that the genes involved in inflammatory responses were mostly upregulated by UVB in *Ogg1*-KO mice, which are susceptible to photocarcinogenesis.

RESULTS

Dietary *S. platensis* inhibited the development and induction of UVB-induced skin tumors

We compared the development of skin tumors induced by chronic exposure to the minimum erythema dose of UVB in *Ogg1*-KO and WT mice fed *S. platensis* (SP(+)) or those not fed *S. platensis* (SP(−)). The total number of skin tumors that developed was significantly higher in the groups of SP(−) mice than those in SP(+); (WT, $P<0.01$; KO, $P<0.00001$). The ratios of malignant tumors/total tumors were not significantly different between SP(−) and SP(+) groups in both genotype mice (Table 1). Histological analysis of all the skin tumors showed that the ratios of malignant tumors were 50 and 66.7% in WT and *Ogg1*-KO mice, respectively, among the skin tumors that developed in the SP(+) group. In the case of the SP(−) group, the ratios of malignant tumors were 50 and 88.5% in WT and *Ogg1*-KO mice, respectively (Table 1). Next, we examined skin tumor induction by chronic UVB exposure in the SP(−) and SP(+) groups, and in each *Ogg1* genotype. As shown in Figure 1, in *Ogg1*-KO mice, the first incidence of skin tumor in SP(−) group was 7 weeks earlier than that in SP(+). After 41 weeks, 100% of *Ogg1*-KO/SP(−) mice harbored skin tumors. In the SP(+) group, by the end of the experiment, we found skin tumors in only 40% of the total population. A similar pattern was observed in the WT mice; the first skin tumor was found during week 37 of the experiment in the WT/ SP(+) group, which is 1 week later than in the WT/SP(−) group. At week 44, only 37.5% of all the WT/SP(+) mice were found to harbor skin tumors, whereas 100% of the WT/SP(−) mice showed tumors (Figure 1). In both genotypes, the incidence of UVB-induced skin tumors was significantly different in these two diet groups. However, in the case of the SP(+) group, there was no significant difference in tumor induction between the different

Table 1. Skin tumor formation by chronic UVB irradiation for each *Ogg1* genotype with or without supplementation of 10% *Spirulina platensis* diet

Ogg1 genotype	*Spirulina platensis* diet	Total number of tumors			Mean number of tumors per mouse	Histological analysis, %				
		Male	Female	Total		Malignant tumors, %			Benign tumor	
						Squamous cell carcinoma	Sarcoma	Total	Papilloma	Unidentified[a]
WT	(+)	2 (2)[b]	2 (6)	4 (8)	0.5 ± 0.76*	50.0 (2/4)[c]	0 (0/4)	50[d] (2/4)	50 (2/4)	0 (0/4)
	(−)	2 (1)	10 (6)	12 (7)	1.71 ± 0.76*	41.7 (5/12)	8.3 (1/12)	50[d] (6/12)	41.7 (5/12)	8.3 (1/12)
KO	(+)	0 (2)	6 (8)	6 (10)	0.6 ± 0.84**	50.0 (3/6)	16.7 (1/6)	66.7[d] (4/6)	16.7 (1/6)	16.7 (1/6)
	(−)	5 (1)	21 (6)	26 (7)	3.71 ± 1.38**	73.1 (19/26)	15.4 (4/26)	88.5[d] (23/26)	11.5 (3/26)	0 (0/26)

Abbreviations: KO, knockout; WT, wild-type.
[a]Unidentified tumors for which we were not able to make the diagnosis due to the failure of the procedure of embedding the specimens.
[b]The number of skin tumor–bearing mice at the end of experiment.
[c]The number of skin tumors/total skin tumors histologically.
[d]Not significant.
*$P<0.01$, **$P<0.0001$. There were 10 mice in each group at the beginning of the experiment; during the experiment some mice died and those were not included.
Data from our previous study for the SP (−) group of WT and *Ogg1*-KO mice were used. (Kunisada *et al.*, 2005).

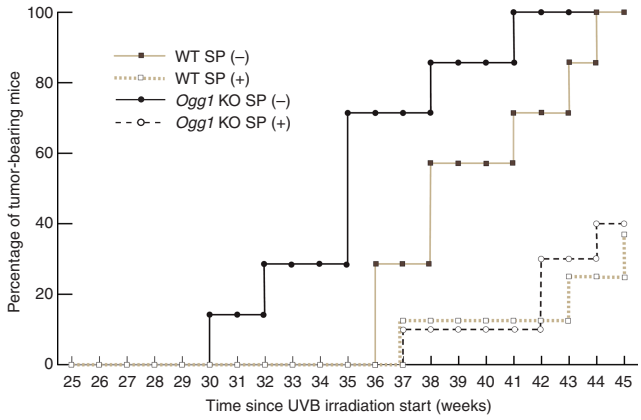


Figure 1. Differences in tumor induction by repetitive UVB irradiations in *Ogg1*-KO and WT mice with or without the *Spirulina platensis* (SP) diet. Each group was composed of 10 mice at the beginning of the experiment. After 40 weeks of chronic UVB exposure, we continued the observation to evaluate tumor development for another 5 weeks. The data for WT/SP(−) and KO/SP(−) mice have been obtained from our previously published study (Kunisada *et al.*, 2005). KO, knockout; WT, wild-type.

Ogg1 genotypes (Figure 1). The data for the SP(−) group of WT mice and *Ogg1*-KO mice have been reproduced here from the results of our previous study (Kunisada *et al.*, 2005). The duration of the experiment and UV irradiation protocols were the same in the present and previous studies and one of the experimenters was involved in both studies. The procedure for chow production was the same with or without *S. platensis*, and was carried out by the same company.

Oral dietary *S. platensis* suppressed UVB-induced ear swelling and skin erythema

We had previously reported that the most upregulated genes in *Ogg1*-KO mice after UVB irradiation were those involved in the inflammatory response, suggesting that the immunomodulation seen after UVB irradiation in *Ogg1*-KO mice might have a role in its highly skin tumor–prone *Ogg1*-KO phenotype (Kunisada *et al.*, 2011). To evaluate the inflammatory response in the skin of mice belonging to the SP(−) or SP(+) group, ear swelling was assessed over a 6-day-period after 4 weeks of oral administration of 10% *S. platensis* followed by a single irradiation of 2.50 kJ m^{-2} UVB to *Ogg1*-KO and WT mice. The peak value for ear swelling was obtained 96 hours after UVB irradiation in all the groups, and significant differences were observed at every time point between SP(−) and SP(+) of each *Ogg1* genotype (WT: $P=0.018$; KO: $P=0.019$; Figure 2a). Next, we studied whether the similar response could be observed in another mouse strain. We applied a protocol similar to that used for *Ogg1*-KO and WT mice to hairless albino mice, except for the UV dose, and then evaluated the erythema scores as well as ear swelling as an inflammatory response upon UVB exposure. A similar inhibitory effect on ear swelling was observed by *S. platensis* in the hairless albino mice: the SP(+) group showed significantly less ear swelling at every time point compared with the SP(−) group after UVB exposure ($P=0.012$; Figure 2b). The erythema on the back skin of hairless albino mice peaked at 72 hours after UVB exposure in both diet groups, with the SP(+) mice showing a much lower degree of erythema and lower amount of scales than the mice belonging to the SP(−) group (Figure 2c). When we scored the degree of erythema, the difference between the SP(−) and SP(+) groups was statistically significant after UVB irradiation ($P=0.011$; Figure 2d).

UVB-induced inflammatory cytokines and Toll-like receptor 4 were attenuated in the skin of *Ogg1*-KO mice fed with *S. platensis*

We had previously reported that IL1-β as well as Cxcl1 is the most important cytokine candidate in the induction of inflammation associated with 8-oxoG accumulation after UVB exposure (Kunisada *et al.*, 2011 and unpublished data). We examined the expression of *IL1-β* and *Cxcl1* after UVB exposure in *Ogg1*-KO and WT mice from the SP(−) and SP(+) groups by using quantitative PCR (qPCR). The difference in interleukin (*IL-1β*) expression between the SP(−) and SP(+) groups in either genotype was most prominent at 24 hours after UVB irradiation. *S. platensis* showed significant inhibition of *IL-1β* expression at all the time points in *Ogg1*-KO mice and at 24 hours after UVB exposure in WT mice ($P<0.05$; Figure 3a). qPCR showed that SP significantly inhibited *Cxcl1* expression at 24 hours after UVB irradiation in *Ogg1*-KO mice and at 6 hours after UVB irradiation in WT mice ($P<0.05$). There was no difference in the expression of *Cxcl1* between the two diet groups for either genotype of mice at other time points (Figure 3b). To confirm the results obtained for *IL1-β* with qPCR, we performed an immunohistochemical study for IL1-β expression in the skin of mice belonging to the SP(−) and SP(+) groups. The epidermal cell expression of IL1-β clearly increased in the *Ogg1*-KO genotype for SP(−) at 24 hours after exposure (Figure 3c). Recently, Toll-like receptor 4 (TLR4) was found to be associated with skin tumorigenesis, via induction of production of proinflammatory cytokines such as IL1-β, IL-6, and tumor necrosis factor-α (Mittal *et al.*, 2010). Our immunohistochemical study showed that TLR4 expression 24 hours after UVB exposure was attenuated in the epidermal cells as well as the dermal inflammatory cells in the SP-treated group in both genotype mice. The attenuation was much more striking in *Ogg1*-KO mice than in WT mice (Figure 3d and e).

Reduction of oxidatively damaged DNA in *S. platensis*–treated mice

We investigated the relevance of UVB-induced formation of epidermal DNA damage such as 8-oxoG and CPDs among the SP(+) and SP(−) groups at 3 and 24 hours after UVB exposure. *Ogg1*-KO/SP(−) mice showed the highest accumulation of 8-oxoG at 3 hours, and even at 24 hours, after UVB exposure. WT mice showed minimal accumulation of 8-oxoG at 3 hours after irradiation, and these levels had decreased almost to zero 24 hours after UVB irradiation. In contrast, both genotypes of the SP(+) group showed less accumulation of 8-oxoG at 3 and 24 hours after UVB exposure (Figure 4a).

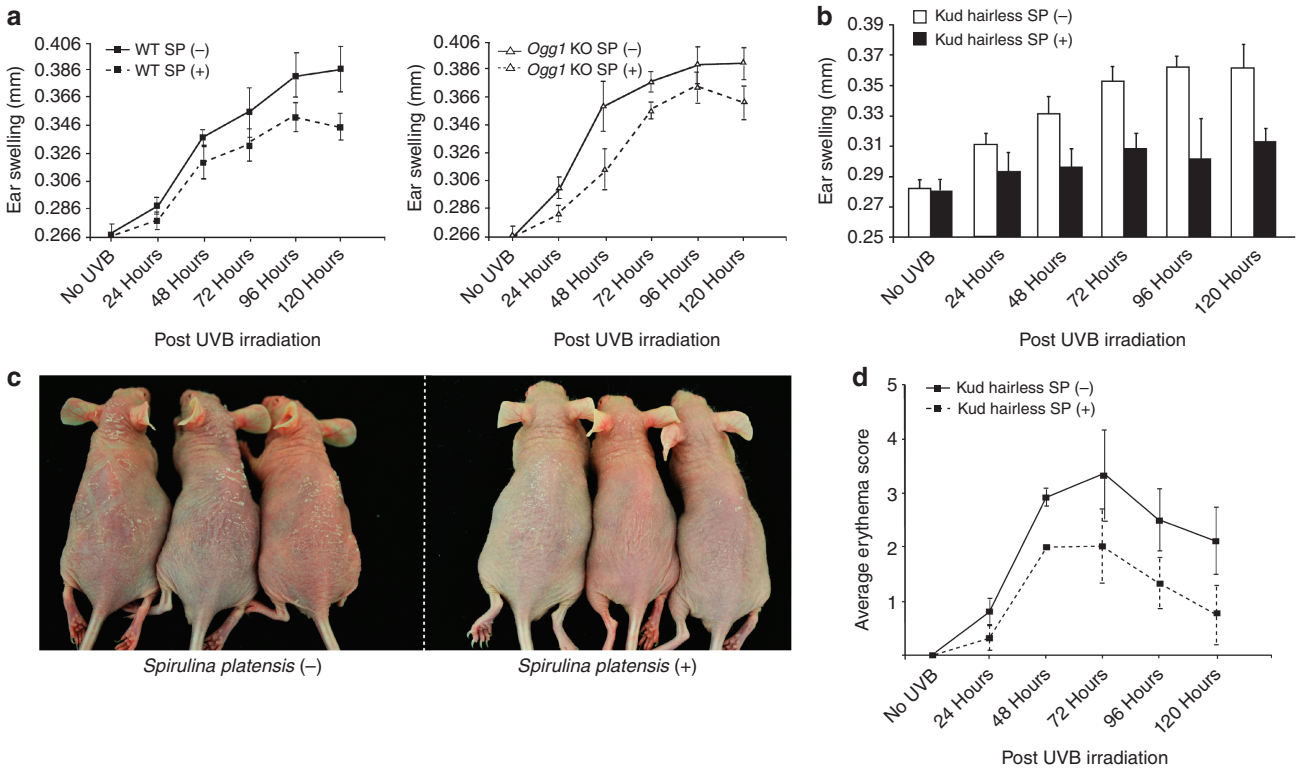


Figure 2. The acute inflammatory response mediated by UVB exposure in mouse skin was attenuated by supplementation with the 10% *Spirulina platensis* (SP) diet. (**a**) After 4 weeks of oral administration of 10% *S. platensis* followed by single irradiation of 2.50 kJ m^{-2} UVB to *Ogg1*-KO and WT mice, ear thickness was measured at 24, 48, 72, 96, and 120 hours after UVB irradiation as well as before UVB treatment. Evaluation of ear thickness was visualized with graphs for WT/SP(−) and WT/SP(+) (left) and for KO/SP(−) and KO/SP(+) (right) mice. Values shown are mean ± SD (**b**) Ear thickness was evaluated using Kud hairless mice given SP(−) and SP(+) diets. The protocol for the hairless mice was identical to that for *Ogg1*-KO and WT mice, except that the UVB dose was 1.50 kJ m^{-2}. Values shown are mean ± SD. (**c**) Erythema induction on the back skin of hairless mice under both SP(−) and SP(+) diets induced by UVB exposure. Before UVB irradiation, hairless mice were fed 10% *S. platensis*. Representative features of the back skin at 72 hours after UVB irradiation in three mice from the SP(−) group (left) and in three mice from the SP(+) group (right), respectively, are shown. (**d**) The average erythema score was measured for the back skin of hairless mice without UVB or at 24, 48, 72, 96, and 120 hours after UVB irradiation. Values shown are mean ± SD. KO, knockout; WT, wild-type.

To confirm whether the UVB dose used for each group was sufficient to cause DNA damage, we performed immunohistochemical analysis of CPDs, showing strongly CPD-positive cells regardless of the ingestion of the *S. platensis* diet or the *Ogg1* genotype (Figure 4b).

Phosphorylation of the stress-activated protein kinases, p38 MAPK, SAPK/JNK, and ERK, was suppressed by *S. platensis*

Furthermore, to investigate the molecular mechanism underlying the suppressive effect of *S. platensis* on the acute inflammatory response mediated by UVB, we examined phosphorylation in the p38 mitogen-activated protein kinase (p38 MAPK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and extracellular signal–regulated kinase (ERK) pathways after a single irradiation with UVB and studied the protein levels at various time points in either *Ogg1*-KO or WT mice from the SP(−) and SP(+) groups by using immunohistochemistry analysis. In WT mice, p38 MAPK phosphorylation and phosphorylation of SAPK/JNK was suppressed at 30 minutes after UVB exposure in the SP(+) group (Figure 5a and b), whereas no obvious differences were observed in the expression of phosphorylated ERK between SP(−) and SP(+) mice at any time point (Figure 5c). In contrast, for *Ogg1*-KO mice, the phosphorylation of p38 MAPK, SAPK/JNK, and ERK was inhibited to a greater extent in SP(+) than in SP(−) mice at every time point after UVB irradiation (Figure 5a–c). Finally, we used *Ogg1*$^{(+/+)}$ and *Ogg1*$^{(-/-)}$ mouse embryonic fibroblasts (MEFs); phycocyanobilin, active form of main functioning protein C-phycocyanin in *S. platensis*, covalently attached to the apoprotein (Figure 5d) was used for specifically analyzing the signal protein kinases after UVB exposure (Schram and Kroes, 1971). In *Ogg1*$^{(+/+)}$ MEFs, no obvious differences were observed in the expression of phosphorylated p38 MAPK between PCB(−) and PCB(+) mice at any time point, whereas phosphorylation of SAPK/JNK and ERK was suppressed at 2 and 6 hours after UVB exposure in the PCB(+) group in *Ogg1*$^{(+/+)}$ (Figure 5e). In contrast, for *Ogg1*$^{(-/-)}$, the phosphorylation of p38 MAPK was inhibited to a greater extent by PCB(+) at 30 minutes after UVB irradiation. SAPK/JNK phosphorylation was also suppressed by PCB(+) in *Ogg1*$^{(-/-)}$at every time point after UVB exposure (Figure 5f). Moreover, at later time points (e.g., 6 and 24 hours), ERK phosphorylation was significantly suppressed in the *Ogg1*$^{(-/-)}$/PCB(+) group (Figure 5f).

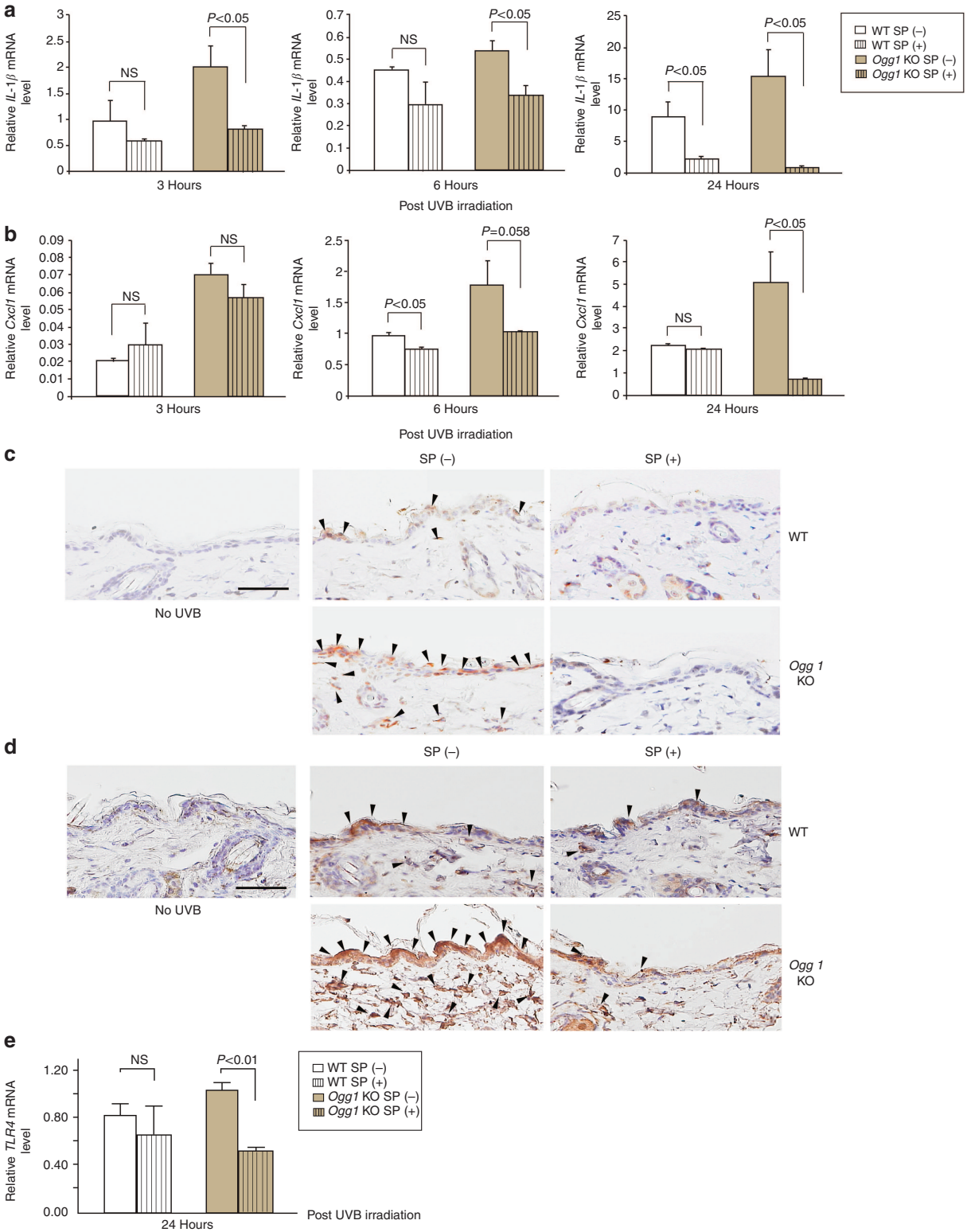


Figure 3. UVB-induced inflammation was attenuated in the skin of *Ogg1*-KO mice that were fed *Spirulina platensis* (SP). The relative mRNA expression of *IL-1β* (**a**) and *Cxcl1* (**b**) in the skin of *Ogg1*-KO and WT mice at different time points after UVB irradiation was determined with two-step real-time quantitative RT-PCR. The expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Values shown are mean ± SD. Immunohistochemical study of IL-1β (**c**) and TLR4 (**d**) expression in *Ogg1*-KO and WT mouse skin. For each group, that is, WT/SP(−), WT/SP(+), KO/SP(−), and KO/SP(+), skin samples were obtained 24 hours after UVB irradiation along with no-UVB control and were subjected to immunohistochemical staining with either rabbit polyclonal antibody against IL-1β or TLR4. Positive cells in the epidermis are indicated by arrowheads. Scale bar = 50 μm. The relative mRNA expression of *TLR4* in *Ogg1*-KO and WT mice 24 hours after UVB exposure was evaluated with quantitative reverse trancriptase–PCR (**e**). IL, interleukin; KO, knockout; TLR4, Toll-like receptor 4; WT, wid-type.

DISCUSSION

Our results showed that UVB-induced skin tumor development was significantly suppressed by dietary *S. platensis* in mice, even though each of our experiments included only a relatively small number of mice per group and used only a historical control group of untreated mice (Kunisada *et al.*, 2005). Epidermal 8-oxoG formation was lower in the SP(+) group than in the SP(−) group, clearly indicating that *S. platensis* exerted a suppressive effect on ROS production caused by UVB in the skin. Indeed, when *S. platensis* is used as a dietary supplement, it increases the levels of antioxidant enzymes and the basal level of reduced glutathione in the tissues (Dasgupta *et al.*, 2001). In particular, the increase of reduced glutathione levels might lead to a decrease in the level of reactive electrophiles able to bind DNA, leading to reduction in ROS-induced 8-oxoG formation. Another significant finding of the present study was the anti-inflammatory effects of *S. platensis* on mouse skin caused by UVB in terms of erythema and ear swelling. Several studies have shown that the expression of the inflammatory cytokine IL-1β is upregulated in UVB-irradiated skin cells (Skiba *et al.*, 2005; Meeran *et al.*, 2009), and we previously reported that the increase in the mRNA expression level of *IL-1β* after UVB exposure was much higher in *Ogg1*-KO mice than in WT mice (Kunisada *et al.*, 2011). In addition, in the present study, *Cxcl1*, a chemokine that functions in neutrophil migration, was also significantly upregulated in *Ogg1*-KO mice after UVB irradiation. Indeed, neutrophil infiltration is a well-known consequence of UVB exposure in mouse skin (Oka *et al.*, 2011) and human skin (Hawk *et al.*, 1988), and infiltrative neutrophils as well as macrophages were more intensively observed in *Ogg1*-KO mice than in WT mice in our previous study (Kunisada *et al.*, 2011). We have also previously shown that significantly increased 3-nitro-L-Y modifications, product mediated by reactive nitrogen species such as peroxynitrite ($ONOO^-$) at inflammation site, were detected after repetitive exposure of mice skin to UVB. Peroxynitrites are generated by the reaction of NO with superoxide (O_2^-), which is released by the infiltrating neutrophils and macrophages (Hattori *et al.*, 1996). Peroxynitrite is known to inactivate important cellular targets and also mediate oxidative damage in DNA, suggesting that phycocyanin from *S. platensis* and phycocyanobilin might scavenge peroxynitrite (Bhat and Madyastha, 2001).

Furthermore, our present data showed that higher TLR4 expression is associated with more severe inflammation induced by UVB/ROS: higher amount of 8-oxoG is associated with higher expression of inflammatory cytokines such as IL-1β and Cxcl1 as well as higher expression of TLR4 (Figures 3d, e and 4). It has been shown that TLR4 is required for skin inflammation–mediated tumorigenesis in a two-stage chemical carcinogenesis model in which the high-mobility group box1 is released from keratinocytes and functions as a ligand for TLR4 (Branco-Madeira and Lambrecht, 2010; Mittal *et al.*, 2010). Our results have shown that *S. platensis* inhibits the expression of IL1-β and TLR4 concomitantly with the inhibition of tumor development, especially inhibitory effect of *S. platensis* was prominent in *Ogg1*-KO mice, which could imply that 8-oxoG might be some trigger signal for the TLR4 expression leading to inflammatory response. In our system, expressions of TLR4 both in the dermal inflammatory cells as well as in the epidermal keratinocytes were inhibited by *S. platensis* resulting in the inactivation of neutrophils and macrophages and leading to the downregulation of Cxcl1 and IL-1β, respectively. The action mechanism of how *S. platensis* inhibited TLR4 in the skin after UVB exposure remains to be elucidated. One possibility is through direct inhibition of TLR4 by *S. platensis* and the other possibility is through its antioxidant pathway resulting in the lower amount of 8-oxoG.

We also investigated which MAPK was actively involved. Our data showed that *S. platensis* effectively inhibited all the analyzed signal protein kinases (i.e., p38 MAPK, SAPK/JNK, and ERK), especially in *Ogg1*-KO mice after UVB exposure. Kim *et al.* reported that p38 MAPK regulates UVB-induced inflammatory responses, presumably through the induction of Cxcl1, IL-6, and cyclooxygenase-2 in the skin of hairless mice (Kim *et al.*, 2005). This finding suggested that *S. platensis* could function as a p38 MAPK inhibitor similar to SB203580, thereby suppressing the production of Cxcl1 and leading to the attenuation of the inflammatory response induced by UVB exposure. In the present study, the phosphorylation of SAPK/JNK was effectively suppressed by *S. platensis* in *Ogg1*-KO and WT mice. As SAPK/JNK is inhibited by the antioxidant *N*-acetylcysteine in human keratinocytes (Assefa *et al.*, 1997), the inhibitory effects of *S. platensis* on the phosphorylation of SAPK/JNK might be attributed to the reduction of ROS production by UVB. Inhibition of the ERK pathway observed in *Ogg1*-KO mice, which regulates the development and progression of cancer (Dhillon *et al.*, 2007) and is involved in UV-induced skin cancers (Li *et al.*, 2012), explains one of its anticancer mechanisms.

In summary, dietary *S. platensis* effectively inhibited UVB-induced skin tumor development and induction in mouse skin through its anti-inflammatory and antioxidant properties. In addition, *S. platensis* also suppressed the phosphorylation of p38 MAPK, SAPK/JNK, and ERK, indicating that it possesses various effective sites for the inhibition of skin tumor development upon UVB exposure. *S. platensis* could be safely and effectively used as a daily dietary supplement for protection against UVB-induced skin tumorigenesis. Our present investigation also supports the epidemiological findings that acute intense exposure to UVR is a risk factor for the development of skin cancers.

MATERIALS AND METHODS

S. platensis dietary food

A fine dark blue-green dried powder containing *S. platensis* and purified phycocyanobilin (PCB), the active form of the main compound C-phycocyanin in *S. platensis*, were kindly provided by DIC LIFTECH (Tokyo, Japan). The purity of C-phycocyanin and phycocyanobilin as estimated by purification with high-performance liquid chromatography was 95 and 85%, respectively. A diet supplemented with 10% *S. platensis* was prepared with standard chow purchased from Clea Japan (Tokyo, Japan). The highest dose of *Spirulina* recommended as health food is 4 g per day for adults with a body weight of 50 kg, and a 10% dose of *S. platensis* is consistent with

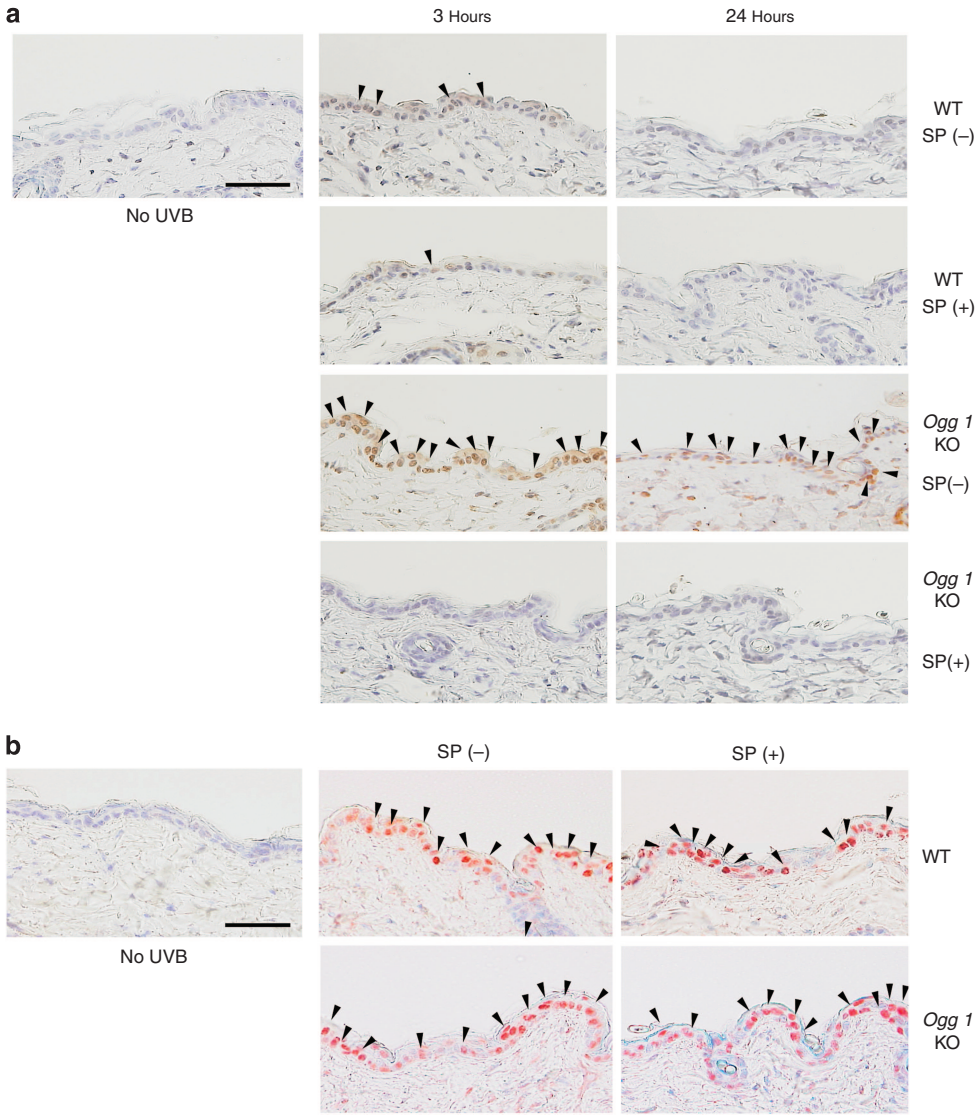


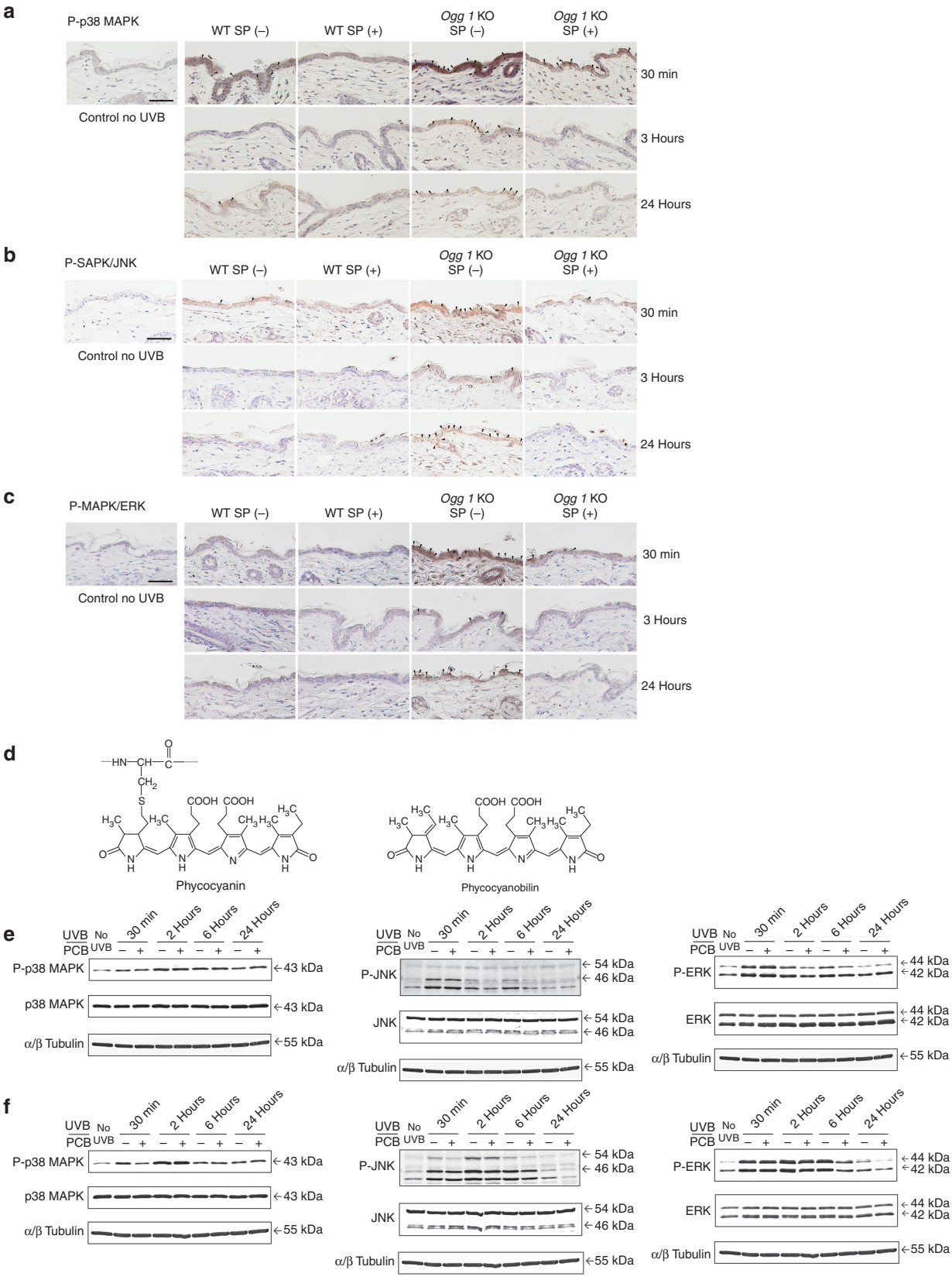
Figure 4. Reduction in oxidatively damaged DNA in the groups of *Spirulina platensis* (SP) treated mice. (**a**) Formation of 8-oxoG in *Ogg1*-KO and WT mouse skin with or without *S. platensis* after UVB exposure. For each group, that is, WT/SP(−), WT/SP(+), KO/SP(−), and KO/SP(+), skin samples were obtained 3 and 24 hours after UVB irradiation along with no-UVB control and subjected to immunohistochemical staining with mouse mAb against 8-oxoG. Positive cells in the epidermis are indicated by arrowheads. Scale bar = 50 μm. (**b**) Immunohistochemical study for CPDs in *Ogg1*-KO and WT mice skin with or without *S. platensis* after UVB exposure. Skin samples from WT/SP(−), WT/SP(+), KO/SP(−), and KO/SP(+) mice were taken 3 hours after UVB irradiation, and immunohistochemical staining was performed using mouse mAb against cyclobutane pyrimidine dimer (TDM-2). 8-oxoG, 8-oxo-7,8-dihydroguanine; KO, knockout; WT, wild-type.

Figure 5. Phosphorylation of stress-activated protein kinases, p38 MAPK, JNK, ERK in *Ogg1*-KO and WT mice was suppressed by *Spirulina platensis*. Immunohistochemical study of phosphorylation of p38 MAPK (**a**), JNK (**b**), and ERK (**c**) in *Ogg1*-KO and WT mice skin. For each group, that is, WT/SP(−), WT/SP(+), KO/SP(−), and KO/SP(+), skin samples were obtained 30 minutes, 3 hours, and 24 hours after UVB irradiation along with no-UVB control and subjected to immunohistochemical staining with rabbit polyclonal antibody against phospho-p38 MAPK, phospho-SAPK/JNK, and phospho-MAPK/ERK. Positive cells are indicated by arrowheads. Scale bar = 50 μm. Phosphorylation of SAPK, p38 MAPK, JNK, and ERK was suppressed in phycocyanobilin-treated $Ogg1^{(+/+)}$ and $Ogg1^{(-/-)}$ MEFs. (**d**) Chemical structure of phycocyanin (left) and phycocyanobilin (PCB; right). PCB was purified and analyzed with high-performance liquid chromatography with tandem mass spectrometry. Immediately after plating, MEFs were incubated with normal medium or 5 μg/ml (8.49 μM) PCB for about 16 hours (overnight), and were then exposed to UVB and incubated again with normal medium or PCB-containing medium until the time of protein extraction (30 minutes, 2 hours, 6 hours, and 24 hours after UVB exposure). The levels of phosphorylation of p38 MAPK, JNK, and ERK in $Ogg1^{(+/+)}$ (**e**) and $Ogg1^{(-/-)}$ (**f**) MEFs were studied by western blot analysis. $Ogg1^{(+/+)}$/PCB(−), $Ogg1^{(+/+)}$/PCB(+), $Ogg1^{(-/-)}$/PCB(−), and $Ogg1^{(-/-)}$/PCB(+). Each experiment is representative of three separate determinations. ERK, extracellular signal–regulated kinase; KO, knockout; MAPK, mitogen-activated protein kinase; MEFs, mouse embryonic fibroblasts; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; WT, wild-type.

previous reports examining the physiological activity of *Spirulina* in rodents (Rodriguez-Hernandez *et al.*, 2001; Ishimi *et al.*, 2006; Lu *et al.*, 2010).

Mice

Ogg1-KO mice were developed as described previously (Sakumi *et al.*, 2003). Mice were selected and divided into two groups:



Ogg1-KO and WT. Next, each genotype group was subdivided into two groups, the *S. platensis* diet (SP(+)) and normal diet (SP(−)) groups. In total, four groups consisting of 10 mice each were irradiated with UVB. To investigate the effect of the *S. platensis* diet on the erythema response, we used Kud^{-} hairless mice, which were divided into two groups, SP(+) and SP(−), and the mice in the SP (+) group were fed the *S. platensis* diet from 4 weeks of age. All the animal experiments were conducted according to the Guidelines for Animal Experimentation of the Kobe University Graduate School of Medicine.

UVB irradiation

A bank of six TL 20W/12RS fluorescent lamps (Philips, Eindhoven, Holland) was used to irradiate the mice as described previously (Kunisada *et al.*, 2005). For skin tumor production, the dorsal parts of the mice were shaved and the mice were placed 40 cm below the light source and irradiated with 2.50 kJ m^{-2} UVB three times per week for 40 weeks. The data for the SP(−) group of WT mice and *Ogg1*-KO mice were reintroduced from the results of our previous study. (Kunisada *et al.*, 2005). The duration of the study was the same between the present and previous studies, and one of the experimenters was involved in both studies. The procedure of chow production was the same with or without *S. platensis* and was carried out by the same company, Clea Japan. For studying the acute response to UVB irradiation, four groups (*Ogg1*-KO/SP(+), *Ogg1*-KO/SP(−), WT/SP(+), and WT/SP(−)) of mice were irradiated with a single dose of 2.50 kJ m^{-2} UVB to measure the ear thickness. For determining the erythema score and ear thickness in Kud Hairless mice, the mice were irradiated with a single dose of 1.50 kJ m^{-2} UVB.

RNA isolation and real-time qPCR

RNA was isolated from mouse dorsal skin at 3, 6, and 24 hours after UVB exposure, as described previously (Kunisada *et al.*, 2011). The expression levels of *IL-1β*, *Cxcl1*, and *TLR4* were analyzed using two-step real-time quantitative reverse transcriptase–PCR (qPCR) using the Universal Probe Library (LightCycler 480 System II; Roche, Mannheim, Germany), according to a previously published protocol (Kunisada *et al.*, 2011). The expression levels of *IL-1β*, *Cxcl1*, and *TLR4* were normalized based on the level of glyceraldehyde-3-phosphate dehydrogenase. All expression levels were again normalized based on 18s ribosomal *RNA* levels to confirm consistency with glyceraldehyde-3-phosphate dehydrogenase normalization (data not shown). Information on the primer sets used for *IL-1β*, *Cxcl1*, and *TLR4* is given in Supplementary Information online.

Immunohistochemical study

Skin specimens were obtained at 30 minutes, 3 and 24 hours after UVB exposure, fixed with 10% neutralized formalin, embedded in paraffin, and used for immunohistochemical staining using mAb against 8-oxoG (N45.1) or cyclobutane pyrimidine dimer (TDM-2) and rabbit polyclonal antibody against IL-1β (Abcam, Cambridge, MA), TLR4 (Abcam), phospho-p38 MAPK (Thr180/Tyr182; Cell Signaling Technology, Beverly, MA), phospho-SAPK/JNK (Thr183/Tyr185; Cell Signaling), and (ERK) 1/2 (Promega, Madison, WI) as described elsewhere (Kunisada *et al.*, 2007, 2011).

Western blot for p38 MAPK, SAPK/JNK, and ERK using *Ogg1* MEFs

Ogg1-KO MEFs, that is, *Ogg1*$^{(-/-)}$ (OG7L), along with WT *Ogg1*$^{(+/+)}$ (OG9L) MEFs were established previously (Oka *et al.*, 2008). MEFs were divided into four groups: *Ogg1*$^{(+/+)}$ treated with 5 μg ml^{-1} PCB, *Ogg1*$^{(+/+)}$ without PCB, *Ogg1*$^{(-/-)}$ treated with 5 μg ml^{-1} PCB, and *Ogg1*$^{(-/-)}$ without PCB. The dose 5 μg ml^{-1} (8.49 μM) is lower than the IC_{50} value of 30.5 ± 0.8 μM, based on the inhibitory effects of peroxynitrite-dependent oxidation in a previous report (Bhat and Madyastha, 2001). All the groups were irradiated with a single irradiation dose of 200 J m^{-2} UVB, and the cells were collected at 30 minutes, 2 hours, 6 hours, and 24 hours after UVB exposure. The blots were incubated with polyclonal antibody against p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), SAPK/JNK, phospho-JNK (Thr183/Tyr185), and alpha/beta-tubulin, which were obtained from Cell Signaling Technology (Beverly, MA), and ACTIVE MAPK and (ERK) 1/2 (Promega, Madison, WI).

Evaluation of average erythema score and ear thickness

After UVB irradiation, evaluation of the erythema and ear thickness in the skin of mice from each group was performed as described previously (Ono *et al.*, 2012). Mean values for the grades were calculated for all the mice at every time point after UVB irradiation.

Statistical study

The differences in the number of developed tumors between the two groups were assessed for significance by using the Student's *t*-test. Statistical differences were examined with the χ^2-test for the malignant tumor rate. Two-factor factorial analysis of variance was applied to differences in ear swelling and erythema scores. $P<0.05$ was considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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