8-Oxoguanine Formation Induced by Chronic UVB Exposure Makes Ogg1 Knockout Mice Susceptible to Skin Carcinogenesis

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Abstract

8-Oxoguanine is one of the oxidative DNA damages that can result in stable mutations. The Ogg1 gene encodes the repair enzyme 8-oxoguanine-DNA glycosylase, which removes the oxidized base from DNA. In this study, we investigated the role of 8-oxoguanine in skin carcinogenesis induced by UVB irradiation using Ogg1 knockout mice (C57Bl/6J background). We examined the effect of UVB irradiation on the formation of 8-oxoguanine in epidermal cells using immunostaining and found that the level of 8-oxoguanine in Ogg1 knockout mice 24 hours after UVB irradiation remained high compared with that in wild-type and heterozygous mice. To verify the effect of chronic UVB irradiation on 8-oxoguanine formations in epidermal cells, we irradiated wild-type, heterozygous, and Ogg1 knockout mice with UVB at a dose of 2.5 kJ/m 2 thrice a week for 40 weeks. We found that the mean number of tumors in Ogg1 knockout mice was 3.71, which was significantly more than in wild-type and heterozygous mice, being 1.71 and 2.28, respectively. The rate of developing malignant tumors in Ogg1 knockout mice was also significantly higher (88.5%; squamous cell carcinomas, 73.1%; sarcomas, 15.4%) than in wild-type mice (50.0%; squamous cell carcinomas, 41.7%; sarcomas, 8.3%). Moreover, the age of onset of developing skin tumors in Ogg1 knockout mice was earlier than in the other types of mice. These results clearly indicate that oxidative DNA damage induced by sunlight plays an important role in the development of skin cancers.

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Introduction

Reactive oxygen species, which are generated endogenously by cellular oxygen metabolism or exogenously by ionizing radiation, environmental mutagens, and carcinogens, produce various types of DNA damage. Among many oxidative DNA base modifications, 8-oxoguanine pairs with adenine as well as cytosine during DNA replication, which results in GC→TA transversion mutations (1, 2). In mammalian cells, Ogg1 encodes a DNA glycosylase/AP lyase, which functions in the removal of 8-oxoguanine from DNA (3). Solar UV, particularly UVB (wavelength range, 280-320 nm), has been recognized to be responsible for the development of skin cancers in humans and in other animals (4). The mechanism of sunlight-related skin carcinogenesis has been extensively investigated and UV-induced pyrimidine photoproducts are thought to be a major cause of skin cancer (5, 6). UV and visible light, on the other hand, are also known to induce reactive oxygen species, and increases in 8-oxoguanine were shown in cells after UVB or visible light exposure (7, 8). In this study, we showed that Ogg1 knockout mice are impaired in the removal of 8-oxoguanine from DNA in epidermal cells after UVB exposure and that Ogg1 knockout mice produce significantly higher numbers of skin tumors after chronic UVB irradiation than do wild-type or heterozygous mice. In addition, we found that the ratio and incidence of malignant skin tumors produced by chronic UVB irradiation in Ogg1 knockout mice were significantly higher and occurred at an earlier age than in wild-type or heterozygous mice. Although there are several reports about the association between the inactivation of Ogg1 and cancer risk (9–11), this study directly clarifies the importance of oxidative DNA damage caused by UVB irradiation in developing skin cancers using Ogg1 knockout mice.

Materials and Methods

Knockout mice. The development of Ogg1 knockout mice has been described previously (9). We inbred Ogg1 heterozygous mice (C57Bl/6J, N:12) and determined three Ogg1 genotypes, wild-type, heterozygous, and knockout, using genomic PCR. The procedure used for PCR, including the position and primers used, has been previously described (9). Mice, ages 12 to 15 weeks, were selected and divided into three groups of 10 mice for Ogg1 genotype, wild-type, heterozygous, and knockout, to be used for UV irradiation. In addition, five mice of each genotype were sham irradiated. The mice were housed under special pathogen-free conditions and all animal experiments were conducted according to the "Guideline for Animal Experimentation at Kobe University School of Medicine."

UVB irradiation. A bank of six T1 TL 20W/12RS fluorescent lamps (Philips, Eindhoven, Holland) was used to irradiate the mice. These lamps emit a continuous spectrum from 275 to 390 nm, with a peak emission at 313 nm; ~ 65% of that radiation is within the UVB wavelength range. The irradiance was 3.8 J/m 2/s at a distance of 40 cm, as measured by an UVR-305/365D digital radiometer (Tokyo Kogaku Kikai KK, Tokyo, Japan). For skin tumor production, the backs of mice were shaved and the mice were placed 40 cm below the light source and irradiated with 2.5 kJ/m 2 UVB thrice per week for 40 weeks. Exposure to 2.5 J/m 2 is an approximate subminimal erythema dose for C57Bl/6J mice. For immunohistochemical detection of 8-oxoguanine after UVB irradiation, mice of age 12 weeks were irradiated with 3.0 kJ/m 2.

Immunohistochemistry. For detection of 8-oxoguanine in mouse skin, skin specimens were collected 3 and 24 hours after UVB irradiation. Skin specimens were fixed in 10% neutralized formalin and embedded in paraffin. Sections were cut, deparaffinized, rehydrated, and washed in PBS. Sections were microwaved thrice in 10 mmol/L citrate buffer (pH 6.0) for 5 minutes. After blocking endogenous peroxidase, nonspecific binding sites were blocked by incubating the sections with protein blocking serum (Dako, Kyoto, Japan). Sections were incubated for 1 hour at 40°C with the primary

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mouse monoclonal antibody against 8-oxoguanine, N45.1 (8). After washing with PBS, the sections were incubated with biotin-conjugated anti-mouse immunoglobulin G (Dako) for 20 minutes at room temperature, followed by incubation for 15 minutes with streptavidin-conjugated horseradish peroxidase (Dako) at room temperature, and finally mounted in glycergel mounting medium (Dako).

Observation and measurement of cumulative tumor incidence. After the chronic UVB exposure, we observed tumor formation until all mice developed skin tumors. The number of developing tumors with diameters larger than 2 mm was counted. Tumors <2 mm in diameter, or regressed, were not counted. The number of tumors of mice that died during the experiment was not included. All mice were sacrificed at the final observation, and all skin tumors were excised and examined histologically with H&E staining. In addition, all mice, including mice that died during the experiment, were subjected to autopsy to confirm whether they had internal spontaneous tumors macroscopically.

Histologic analysis. Biopsied tumors were fixed with 10% neutralized formalin, embedded in paraffin, and then stained using H&E. Tumors examined histologically were classified as squamous cell carcinomas (tumors with atypical epithelial differentiation), sarcomas (tumors with atypical mesenchymal differentiation), papillomas (tumors with papillomatous growth of epidermal cells without atypicality), or hemangiomas.

Statistical analysis. Statistical differences were examined with unpaired t test for mean number of tumors per mouse and with χ² test for malignant tumor rate; P < 0.05 is considered to be statistically significant.

Results and Discussion

8-Oxoguanine can be generated via a variety of agents such as chemicals, X-radiation, and UV and visible light in the presence of a photosensitizer, and 8-oxoguanine is accepted as a sensitive marker of oxidative DNA damage (3, 12). 8-Oxoguanine induces GC—TA transversions by mispairing with A as well as C. GC—TA transversions have been observed in the ras and p53 genes in UVB-induced mice skin cancers as well as in human non-melanoma skin cancers of sun-exposed areas, which implies that 8-oxoguanine plays an important role in UV-related skin carcinogenesis (6, 13, 14). Previously it has been shown that UVB exposure increases the amount of 8-oxoguanine formations in epidermal cells (8). We examined the time course of relative amounts of 8-oxoguanine in epidermal cells of each Ogg1 genotype immunohistochemically using monoclonal antibody against 8-oxoguanine (Fig. 1). Few cells positively stained for 8-oxoguanine were seen in the epidermis of sham-irradiated skin, as expected. Skin specimens from UVB-irradiated wild-type and heterozygous mice showed similar staining patterns, where positively stained nuclei were seen 3 hours after UVB irradiation, although most of which waned to the level of sham-irradiated mice 24 hours after UVB irradiation. These data, however, are inconsistent with a

![Figure 1](image-url)
previous report, which indicated that UV-induced 8-oxoguanine formations in hairless mouse skin were slowly removed and remained at a high level in the epidermis (12). These data with the previous reports were achieved because the dose of UVB irradiation used in this study was relatively low (subminimal erythema dose) compared with the much higher dose used in the other study, which was 10 times the minimal erythema dose (12). Yet another consideration might be that the mice used in this study were shaved hairy black mice whereas those used in the other study were albino hairless mice. Shading by the remaining hair and pigment in the skin of the mice used in this study might have reduced the direct damage to epidermal cells to some extent. Another explanation is that the difference in mice strains contributes to the variety of skin carcinogenesis, because the C57Bl background mice we studied are well known for their resistance to carcinogen (15). Consequently, the majority of 8-oxoguanine in epidermal cells of Ogg1 heterozygous and wild-type mice was removed within 24 hours. The epidermal cells of Ogg1 knockout mice, on the contrary, remained highly positive for 8-oxoguanine in the epidermis, even 24 hours after UVB irradiation, similar to the level observed 3 hours after UVB irradiation. This indicates that Ogg1 knockout mice have impairment in removing 8-oxoguanine from epidermal cells. Another report showed similar results that Ogg1 knockout mice have a decreased ability to repair 8-oxoguanine in the liver after KBrO3 treatment (16). Therefore, it is reasonable to speculate that cumulative 8-oxoguanine in the skin of Ogg1 knockout mice leads to a higher susceptibility to tumorigenesis. Indeed, we found that the mean number of tumors per mouse that developed in Ogg1 knockout mice was significantly (2.2-fold) higher than that in wild-type mice (3.71 versus 1.71, respectively; \( P < 0.01 \)) after 120 times UVB exposure of 2.5 kJ/m²/time over 40 weeks (Table 1; Fig. 2). The mean number of tumors per mouse for the Ogg1 knockout mice was also significantly (1.6-fold) higher than that of the heterozygous mice (3.71 versus 2.28, respectively; \( P < 0.02 \); Table 1). There was no significant difference between wild-type and heterozygous mice in the mean number of tumors per mouse. These findings are consistent with the 8-oxoguanine staining patterns among the three groups at 24 hours after UVB irradiation (Table 1; Fig. 1). These results show the first direct evidence that the persistent presence of 8-oxoguanine following UVB exposure is closely related to the development of skin tumors. We did histologic analysis for all skin tumors developed. Among skin tumors produced in Ogg1 knockout mice, 88.5% were malignant tumors (squamous cell carcinomas, 73.1%; sarcomas, 15.4%), which is a significantly higher rate than that produced in wild-type mice, in which 50.0% were malignant tumors (squamous cell carcinomas, 41.7%; sarcomas, 8.3%; \( P < 0.05 \); Table 1). Because there are some reports indicating that the spindle cell tumors, which appear to be sarcoma, have the characteristics of keratinocyes in terms of immunohistochemical and ultrastructural findings (17), we examined malignant tumors consisted mostly of spindle cells immunohistochemically with pan-cytokeratin and found that none of the six specimens examined showed positive staining for pan-cytokeratin (data not shown). Thus, we diagnosed these tumors as sarcoma in this study. Furthermore, we evaluated the acceleration of developing tumors caused by Ogg1 gene disruption. As shown in Fig. 3,

**Table 1. Skin tumor formation by chronic UVB irradiation for each Ogg1 genotype mice**

<table>
<thead>
<tr>
<th>Ogg1 genotype</th>
<th>UVB irradiation</th>
<th>Total number of tumors</th>
<th>Mean number of tumors per mouse</th>
<th>Histological analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
<td>Malignant tumors (%)</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>2 (1)</td>
<td>10 (6)</td>
<td>12 (7)</td>
<td>1.71 ± 0.76 (^{1,2})</td>
</tr>
<tr>
<td></td>
<td>(5/12)</td>
<td>(1/12)</td>
<td>(6/12)</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>0 (1)</td>
<td>0 (4)</td>
<td>0 (5)</td>
<td>0</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>4 (1)</td>
<td>12 (6)</td>
<td>16 (7)</td>
<td>2.28 ± 0.76(^{1,2})</td>
</tr>
<tr>
<td></td>
<td>(10/16)</td>
<td>(1/16)</td>
<td>(11/16)</td>
<td></td>
</tr>
<tr>
<td>Knockout</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>5 (1)</td>
<td>21 (6)</td>
<td>26 (7)</td>
<td>3.71 ± 1.38(^{1,2})</td>
</tr>
<tr>
<td></td>
<td>(19/26)</td>
<td>(4/26)</td>
<td>(23/26)</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>0 (1)</td>
<td>0 (4)</td>
<td>0 (5)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Unidentified tumors were those which we were not able to make the diagnosis due to the failure of the procedure of embedding the specimens.

\(^{1}\)The number of skin tumor–bearing mice at the end of the experiment.

\(^{2}\)P < 0.01.

\(^{A}\)P, not significant.

\(^{1}\)The number of skin tumors/total skin tumors histologically.

\(^{*}\)P < 0.05.

\(^{**}\)P < 0.02.
Ogg1 knockout mice began to develop tumors 6 and 5 weeks earlier than wild-type and heterozygous mice, respectively, and 100% incidence was reached at 41 weeks, 3 weeks earlier than in the other genotypes. The age of onset of developing tumors in Ogg1 heterozygous mice revealed a similar pattern to the wild-type mice. Oxidative stress has been shown to be associated with multistage carcinogenesis—initiation, promotion, and progression (18). It is recognized that low levels of oxidants can modify cell-signaling proteins. Thus, it is possible that the higher rate of DNA damage accumulation in Ogg1 knockout mice caused higher frequency of DNA mutation leading to early initiation and acceleration of tumor progression. This would explain our result showing a higher ratio of malignant tumors and earlier onset of tumor formations in Ogg1 knockout mice compared with wild-type and heterozygous mice. Finally, after the final observation, we did autopsies of all mice, including those that died during the experiment, to confirm whether they had internal spontaneous tumors macroscopically (9), but we did not identify any evident internal tumors in any mice.

Several skin cancer–prone diseases, such as xeroderma pigmentosum, Bloom syndrome, and Werner syndrome, are known to have defects in DNA repair systems or in DNA replication systems, such as nucleotide excision repair, translesional pathway, and helicase. Repair deficiencies cause mutations, and mutations in crucial oncogene-related genes result in the development of cancers. Thus, it is reasonable that disruption of both alleles of Ogg1 causes a skin cancer–prone phenotype (19).

We could consider another possible mechanism for the susceptibility of Ogg1 knockout mice to skin cancers. Not only the direct attack of UVB to DNA but also the chronic inflammatory state following UV irradiation is also responsible for the formation of 8-oxoguanine (6, 8). Peroxinitrite is generated through the reaction of nitric oxide with superoxide, which is released by infiltrating neutrophils and macrophages. Reactive oxygen species derived from inflammatory cells are continuously formed. There could be another possibility that higher amount of oxidative stress accelerates epidermal hyperplasia. However, we found that there was no significant difference in the epidermal thickness among the three genotypes.
at least 24 hours after single UVB exposure, although there were subtle variations in epidermal thickness among the specimens independent of the genotype. In summary, UVB exposure generates 8-oxoguanine in epidermal cells, and Ogg1 knockout mice have a higher susceptibility to skin cancers following chronic UVB irradiation. Oxidative DNA damage caused by UVB exposure, as well as pyrimidine photoproducts, has an important contribution in the development of skin cancers.

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References

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