Enhancement of ultraviolet B-induced apoptosis and elimination of DNA damage by pre-irradiation with infrared radiation A does not depend on DNA damage repair

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Running Title: Effect of IRA on DNA repair and apoptosis

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ABSTRACT

Background: We previously reported that pre-irradiation with infrared radiation A (IRA) eliminated ultraviolet B (UVB)-induced cyclobutane pyrimidine dimers (CPDs). Accelerated elimination of CPDs could have resulted from enhanced DNA repair and/or enhanced induction of apoptosis. In this study, we examined whether IRA accelerated the elimination of CPDs by enhancing DNA repair, using Xpa knockout (KO) mice, which were deficient in DNA repair.

Methods: We have already generated mice harboring epidermal melanocytes that produce only eumelanin and dominant pheomelanin, and no melanin. To obtain such mice but with impaired DNA repair ability, we backcrossed these mice with Xpa KO mice. Three hours before UVB irradiation, the mice were irradiated with IRA, and CPDs and apoptotic cells were examined.

Results: Pre-irradiation of Xpa KO mice with IRA before UVB irradiation could accelerate the removal of CPDs and enhance apoptotic changes.

Conclusion: These results indicate that the enhancement of UVB-induced apoptosis and acceleration of removal of CPDs by pre-irradiation with IRA does not depend on DNA damage repair.

Key Words: infrared radiation A, DNA repair, apoptosis, cyclobutane pyrimidine dimer, UV
INTRODUCTION

Infrared radiation A (IRA) has wavelengths in the range of ~760-1400 nm and penetrates human skin.\(^1\) IRA is often used for treating wrinkles and diabetic skin ulcers.\(^2\) Therefore, the safety of repetitive irradiation with IRA needs to be clarified.

IRA is absorbed by melanin and generates heat. Therefore, to examine the effect of IRA on mouse skin, specific mice that harbor melanocytes in the epidermis should be recruited. For this purpose, we previously generated hairless mice harboring epidermal melanocytes that synthesize eumelanin (black hairless (BHSCF) mice) and pheomelanin (yellow hairless (YHSCF) mice), and no melanin (white hairless (WHSCF) mice) by backcrossing K14-stem cell factor (SCF) mice and recessive yellow mice with albino hairless mice.\(^3\) Eighteen-week irradiation with IRA thrice a week on photo-aged mice, which were prepared by repetitive irradiation with ultraviolet B (UVB; ~290-320 nm) thrice a week for 14 weeks, did not enhance tumor formation but rather suppressed it, especially in the eumelanin-harboring mice.\(^3\)

UVB irradiation is known to induce DNA damage, including the formation of cyclobutane pyrimidine dimers (CPDs). We previously reported that pre-irradiation with IRA accelerated elimination of UVB-induced CPDs and enhanced apoptosis. Accelerated removal of CPDs can be induced either by enhanced repair of CPDs or enhanced apoptotic changes. UVB-mediated DNA damage is repaired by nucleotide excision repair (NER). Xpa (xeroderma pigmentosum A) knockout (KO) mice are deficient in NER.\(^4\) To determine whether NER is involved in the reduction in the level of CPDs upon pre-irradiation with IRA, we generated BHSCF, YHSCF, and WHSCF mice with impaired DNA repair ability by backcrossing these mice with Xpa KO mice. Using these mice, we examined the effect of IRA on the elimination of UVB-induced
CPDs and apoptotic changes to determine whether it depended on enhanced DNA repair.

MATERIALS AND METHODS

Mice
We previously generated hairless mice harboring epidermal melanocytes that produce only eumelanin (BHSCF) or pheomelanin (YHSCF), and not melanin (WHSCF). To generate mice with impaired DNA repair ability, we crossed BHSCF, YHSCF, and WHSCF mice with Xpa KO mice (gifted by Dr. Tanaka, Osaka University). Xpa\(^{+/+}\) (Xpa wild), Xpa\(^{+/-}\), or Xpa\(^{-/-}\) (Xpa KO) mice, were determined by genotyping. These mice were fed a standard diet and water, and supported at controlled temperature and humidity with a 12 h light/dark cycle in our University Animal Facility. This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Nippon Medical School Animal Experimentation Regulations (24-159).

Irradiation of mice
IRA was irradiated using a water-filtered IRA irradiation source (Hydrosun-Strahler 505, Hydrosun Medizintechnik, Müllheim, Germany) equipped with an RG830 filter (Shibuya Kogaku, Saitama, Japan) emitting light in the range of 830-1400 nm. Mice were irradiated at a distance of 12 cm in containers placed on ice, thereby avoiding death by heat generated due to IRA irradiation. IRA was irradiated at doses of 135, 360, and 720 J/cm\(^2\) prior to UVB irradiation. These doses were previously used for Xpa wild mice, and they did not induce the formation of CPDs and apoptotic changes.

For UVB irradiation, a bank of four fluorescent UVB lamps (FL20SE/MDR, Toshiba, Tokyo) emitting in the wavelength range of 280-370 nm (mainly UVB energy with a
peak at 305 nm) was used. UVB was irradiated on WHSCF/Xpa KO, YHSCF/Xpa KO, and BHSCF/Xpa KO mice at 0.06, 0.10, and 1.0 J/cm², respectively. These doses were determined using four minimum erythematous doses (MEDs). MEDs of each Xpa KO mice were one fifth of those of XPA wild mice. Skin specimens were taken 15 min, 3 h, 24 h, and 72 h after UVB irradiation.

**Histological analysis**

Biopsied skin was fixed in 4% buffered paraformaldehyde and embedded in paraffin. As previously reported, for immunostaining of CPDs, sections were de-paraffinized and washed in phosphate-buffered saline, and endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in distilled water for 15 min; then, the sections were treated with 2 M HCl for 30 min for DNA denaturation. Blocking of non-specific staining due to anti-mouse IgG was done by applying an M.O.M. (mouse on mouse) kit (Vector Labs, Burlingame, CA, USA) according to the manufacturer’s instructions. The sections were incubated at 4 °C overnight with antibodies against CPDs (1:3000 dilution) (Cosmo Bio, Tokyo, Japan). Staining was performed using an indirect immunoperoxidase technique. Owing to the presence of heavy melanin, we visualized the products in red color using Nova RED (Vector Labs) as the substrate instead of diaminobenzidine (DAB), and the slides were counterstained for nuclei with hematoxylin. For negative control, mouse IgG was used.

For terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays, a previously reported methodology was followed. Briefly, sections were de-paraffinized and washed in phosphate-buffered saline, and then incubated first with proteinase K (20 μg/ml) for 15 min at room temperature and quenched in 0.3% hydrogen peroxide in distilled water for 5 min. The sections were
labeled with a biotin-dUTP mixture using TdT enzyme (In situ Apoptosis Detection Kit, TaKaRa Co. Ltd., Osaka, Japan). The slides were incubated at 37 °C for 90 min, treated with anti-fluorescein isothiocyanate (FITC) horseradish peroxidase (HRP) conjugate for 30 min at 37 °C, and visualized using Nova RED substrate. The TUNEL-positive cells were examined under a light microscope.

Statistical analysis

All values were expressed as means ± standard deviations (SD). Two-way analysis of variance (ANOVA) followed by the Tukey test was used to evaluate differences between more than three groups. Differences were considered to be significant for p values < 0.05.

RESULTS

Effect of pre-irradiation with IRA on UVB-induced DNA damage in Xpa KO mice

The skin samples taken from mice 15 min, 3 h, 24 h, and 72 h after UVB irradiation with or without pre-irradiation with IRA were assessed for CPD positivity. The percentage of CPD-positive cells per 400 epidermal keratinocytes other than adnexal epithelia was calculated. The average percentage of 3 mice of each irradiation group was calculated. For WHSCF/Xpa KO mice, the percentages of CPD-positive cells under the conditions of UVB alone, and UVB and IRA irradiation at doses of 135, 360, or 720 J/cm² were 62.5%, 60.0%, 58.7%, and 57.5% at 15 min; 61.0%, 58.8%, 55.6%, and 55.0% at 3 h; 18.2%, 13.1%, 15.0%, and 13.3% at 24 h; and 3.8%, 3.3%, 2.1%, and 2.0% at 72 h, respectively (Fig. 1a). For YHSCF/Xpa KO mice, the percentages were 61.6%, 63.7%, 62.3%, and 62.3% at 15 min; 55.5%, 54.6%, 51.5%, and 56.0% at 3 h; 43.1%, 33.3%, 36.1%, and 35.0% at 24 h; and 7.2%, 5.5%, 4.3%, and 4.1% at 72 h,
respectively (Fig. 1b). For BHSCF/Xpa KO mice, the percentages were 68.3%, 69.8%, 68.1%, and 69.8% at 15 min; 60.2%, 58.4%, 56.3%, and 56.4% at 3 h; 21.1%, 13.2%, 15.1%, and 12.9% at 24 h; and 3.3%, 2.8%, 2.5%, and 2.3% at 72 h, respectively (Fig. 1c). Fifteen minutes after UVB irradiation, the percentage of CPD-positive cells was approximately 60% in all the groups of mice. Pre-irradiation with IRA accelerated the removal of CPD-positive cells in all skin types of mice, and this was statistically significant in BHSCF mice (Fig. 1d).

Effect of pre-irradiation with IRA on UVB-induced apoptosis in Xpa KO mice

The percentage of positive cells was calculated using the TUNEL assay for 3 mice of each irradiation group. For WHSCF/Xpa KO mice, the percentages of apoptotic cells under the conditions of UVB alone, and UVB and IRA irradiation at doses of 135, 360, or 720 J/cm² were 20.1%, 27.9%, 34.8%, and 38.2% at 24 h, respectively (Fig 2a). For YHSCF/Xpa KO mice, the percentages were 24.8%, 35.0%, 38.1%, and 38.2% at 24 h, respectively (Fig. 2b). For BHSCF/Xpa KO mice, the percentages were 27.1%, 46.7%, 47.1%, and 45.2% at 24 h, respectively (Fig. 2c). Pre-irradiation with IRA enhanced the apoptotic cell number in all skin types of mice, and it was statistically significant. The percentage of apoptotic cells was highest in the BHSCF/Xpa KO mice (Fig. 2d).

DISCUSSION

Using Xpa KO mice, we have shown that pre-irradiation with IRA can help eliminate CPDs quickly. These results are similar to those obtained using Xpa wild mice. This indicates that the effect of IRA on the elimination of CPDs does not involve the enhancement of DNA repair. Another pathway to eliminate CPDs could have been the enhancement of the apoptotic process. Pre-irradiation with IRA significantly increased
the number of TUNEL-positive cells in all skin types, and this effect was especially remarkable in BHSCF mice harboring eumelanin in the epidermis. Similar results were reported by Yamaguchi et al. for human skin.\textsuperscript{5} They showed that UVB-induced apoptosis takes place more in dark skin than in fair skin by inducing the phosphorylation of Ser46 in p53.\textsuperscript{5} The incidence of photocarcinogenesis is known to be much lower in dark skin type compared with that in fair skin type. One well known possibility is that eumelanin protects DNA damages. From the results of ours and Yamaguchi et al., enhanced apoptotic changes of DNA damaged cells by IRA as well as UVB might result in elimination of mutated cells. Schroeder \textit{et al.} reported that IRA irradiation significantly decreased the antioxidant content of skin within 5 minutes after exposure and 24 hours post irradiation, skin antioxidant capacity fully recovered.\textsuperscript{1} The effect of IRA on skin cells seems to appear within several minutes and disappear within 24 h. In fact, for the wound healing of diabetic ulcers, Horwitz et al. irradiated IRA every day for 2 weeks to 11 months.\textsuperscript{6}

UVB-induced apoptosis is a complex process involving the activation of p53 by nuclear DNA damage, release of cytochrome C from mitochondria, and activation of death receptors upon receptor clustering.\textsuperscript{7} In our previous study, real-time PCR analysis indicated that pre-irradiation with IRA reduced UVB-induced suppression of the mRNA expression of the anti-apoptotic genes FLIP\textsubscript{L} and BCL-X\textsubscript{L}, and the induction of the apoptotic gene BAX.\textsuperscript{4} These results indicated that pre-irradiation with IRA could suppress UVB-induced apoptotic changes, considering the role of FLIP\textsubscript{L}, BCL-X\textsubscript{L}, and BAX. However, our current results show that pre-irradiation with IRA enhances UVB-induced apoptosis.

One possible pathway for the enhancement of UVB-induced apoptosis by
pre-irradiation with IRA may be its effect on mitochondria. Mitochondria contain trace metals such as iron and copper, which can be chromophores of IRA. Calles et al. reported a microarray analysis of cultured human fibroblasts showing that genes in the categories of extracellular matrix, calcium homeostasis, stress signaling, and apoptosis were modified by IRA irradiation. Furthermore, they showed that a major part of the responsive genes of IRA was triggered by mitochondria. Therefore, the accelerated elimination of CPDs upon enhancement of apoptosis by pre-irradiation with IRA could have resulted from the action of IRA on mitochondria. To clarify the effects of IRA irradiation on the apoptotic pathway in melanin-burden keratinocytes, we are currently performing microarray analysis using cultured eumelanin-containing keratinocytes established from BHSCF mice (Okazaki et al., manuscript in preparation).

In summary, using mice generated with the background of Xpa KO mice, we have shown that the enhancement of UVB-induced apoptosis and acceleration of removal of CPDs by pre-irradiation with IRA does not depend on DNA damage repair. A detailed study on the pathway of enhancement of apoptotic changes by pre-irradiation with IRA might be needed to better understand the effect of IRA on melanin-burden skin.
**Acknowledgments:** None.
Conflict of interest

The authors have no conflict of interest to declare.
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**Figure legends**

**Figure 1.** Effect of pre-irradiation with infrared radiation A (IRA) on ultraviolet B (UVB)-induced cyclobutane pyrimidine dimer (CPD) formation. IRA was irradiated 3 h prior to UVB irradiation on WHSCF/Xpa KO mice, YHSCF/Xpa KO mice, and BHSCF/Xpa KO mice at doses of 135, 360, and 720 J/cm², respectively. Then, UVB was irradiated at doses of 0.06, 0.1, and 1.0 J/cm² on WHSCF/Xpa KO mice, YHSCF/Xpa KO mice, and BHSCF/Xpa KO mice, respectively. Skin specimens were taken 15 min, 3 h, 24 h, and 72 h after UVB irradiation. Immunostaining for CPD was performed. (a) WHSCF/Xpa KO mice, (b) YHSCF/Xpa KO mice, and (c) BHSCF/Xpa KO mice. (d) Graph showing percentage of CPD-positive keratinocytes 24 h after UVB irradiation. Data are expressed as means ± SD of results of three independently irradiated mice. *p < 0.05 (versus UVB alone).

**Figure 2.** Effect of pre-irradiation with infrared radiation A (IRA) on ultraviolet B (UVB)-induced apoptotic change. IRA and UVB were irradiated at doses similar to those shown in Fig. 1. TUNEL assay was performed. (a) WHSCF/Xpa KO mice, (b) YHSCF/Xpa KO mice, and (c) BHSCF/Xpa KO mice. (d) Graph showing percentage of positive keratinocytes 24 h after UVB irradiation. Data are expressed as means ± SD of results of three independently irradiated mice. *p < 0.05 (versus UVB alone).
Fig. 1  Effect of pre-irradiation of IRA on UVB-induced CPD formation

a.  

b.  

c.  

d.  

![Graph showing CPD-positive keratinocytes](image)
Effect of pre-irradiation of IRA on UVB-induced apoptotic change.

Fig. 2

a.

UVB
IRA 135J/cm²+UVB
IRA 360J/cm²+UVB
IRA 720J/cm²+UVB

b.

UVB
IRA 135J/cm²+UVB
IRA 360J/cm²+UVB
IRA 720J/cm²+UVB

c.

UVB
IRA 135J/cm²+UVB
IRA 360J/cm²+UVB
IRA 720J/cm²+UVB

d.

Bar graph showing TUNEL-positive keratinocytes (%)

- WHSCF 24h
- YHSCF 24h
- BHSCF 24h

Legend:
- UVB
- UVB+IRA135J/cm²
- UVB+IRA360J/cm²
- UVB+IRA720J/cm²

* Indicates statistical significance.