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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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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. Using *Xpa* knockout (KO) mice, which are deficient in DNA repair, we examined whether IRA accelerated elimination of CPDs by enhancing 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 with impaired DNA repair ability, we backcrossed them 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 accelerated removal of CPDs and enhanced apoptotic changes.

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

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

Introduction

Infrared radiation A (IRA) has wavelengths in the range of ～760-1,400 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 are required. For this purpose, we previously generated hairless mice harboring epidermal melanocytes that synthesize eumelanin (black hairless [BHSCF] mice) and pheomelanin (yellow hairless [YHSCF] mice) but 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 eumelanin-harboring mice[3].

UVB irradiation induces DNA damage, including formation of cyclobutane pyrimidine dimers (CPDs). We previously reported that pre-irradiation with IRA accelerated elimination of UVB-induced CPDs and enhanced apoptosis. Removal of CPDs can be accelerated 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 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

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mice, we then examined the effect of IRA on 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) but not melanin (WHSCF)[3]. 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)[4]. $Xpa^{+/+}$(*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 830-1,400 nm[3]. Mice were irradiated at a distance of 12 cm in containers placed on ice, thereby avoiding death by heat generated from IRA irradiation[3]. IRA was irradiated at doses of 135, 360, and 720 J/cm^2 before UVB irradiation. These doses were previously used for *Xpa* wild mice and did not induce formation of CPDs and apoptotic changes[3].

For UVB irradiation, a bank of 4 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[3]. UVB was irradiated on WHSCF/*Xpa* KO, YHSCF/*Xpa* KO, and BHSCF/*Xpa* KO mice at 0.06, 0.10, and 1.0 J/cm^2, respectively. These doses were determined by using 4 minimum erythematous doses (MEDs). MEDs of each *Xpa* KO mice were one fifth those for *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[3], for immunostaining of CPDs, sections were deparaffinized 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 using 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 (1:3,000 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 by 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 dUTP nick-end labeling (TUNEL) assays, a previously reported method was followed[3]. Briefly, sections were deparaffinized 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 ± SD. Two-way analysis of variance (ANOVA) followed by the Tukey test was used to evaluate differences between more than 3 groups. Differences were considered to be significant for p values of <0.05.

Results

Effect of Pre-irradiation with IRA on UVB-induced DNA Damage in *Xpa* KO Mice

Skin samples taken from mice at 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 for 3 mice from each irradiation group was calculated. For WHSCF/*Xpa* KO mice, the percentages of CPD-positive cells under conditions of UVB alone, and UVB and IRA irradiation at doses of 135, 360, or 720 J/cm^2 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 groups of mice. Pre-irradiation with IRA accelerated removal of CPD-positive cells in all mouse skin types, 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 conditions of UVB alone, and UVB and IRA irradiation at doses of 135, 360, or 720 J/cm^2, 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 mouse skin types and the differences were 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[3]. This indicates that the effect of IRA on elimination of CPDs does not involve enhancement of DNA repair. Another potential pathway in eliminating CPDs is enhancement of apoptosis. Pre-irradiation with IRA significantly increased the number of TUNEL-positive cells in all skin types, and this effect was dramatic in BHSCF mice harboring eumelanin in the epidermis. Similar results were reported for human skin by Yamaguchi et al[5]. They showed that UVB-induced apoptosis takes place more in dark skin than in fair skin by inducing phosphorylation of Ser46 in p53[5]. The incidence of photocarcinogenesis is much lower in dark skin than in fair skin. One well known possibility is that eumelanin protects against DNA damages. The present results and those of Yamaguchi et al. suggest that enhanced apoptotic changes of DNA damaged cells by IRA, as well as UVB, might result in elimination of mutated cells. Schroeder et al. reported that IRA irradiation significantly decreased the antioxidant content of skin within 5 min after exposure and that 24 h after irradiation skin antioxidant capacity fully recovered[1]. The effect of IRA on skin cells seems to appear within several minutes and disappear within 24 h. In fact, to hasten wound healing of diabetic ulcers, Horwitz et al. irradiated IRA every day for 2 weeks to 11 months[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[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 $FLIP_L$ and $BCL\text{-}X_L$, and the induction of the apoptotic gene BAX[4]. These results indicate that pre-irradiation with IRA could suppress UVB-induced apoptotic changes, considering the role of $FLIP_L$, $BCL\text{-}X_L$, and BAX. However, our current results show that pre-irradiation with IRA enhances UVB-induced apoptosis.

One possible pathway for 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 that 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[8]. Therefore, 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. To better understand the

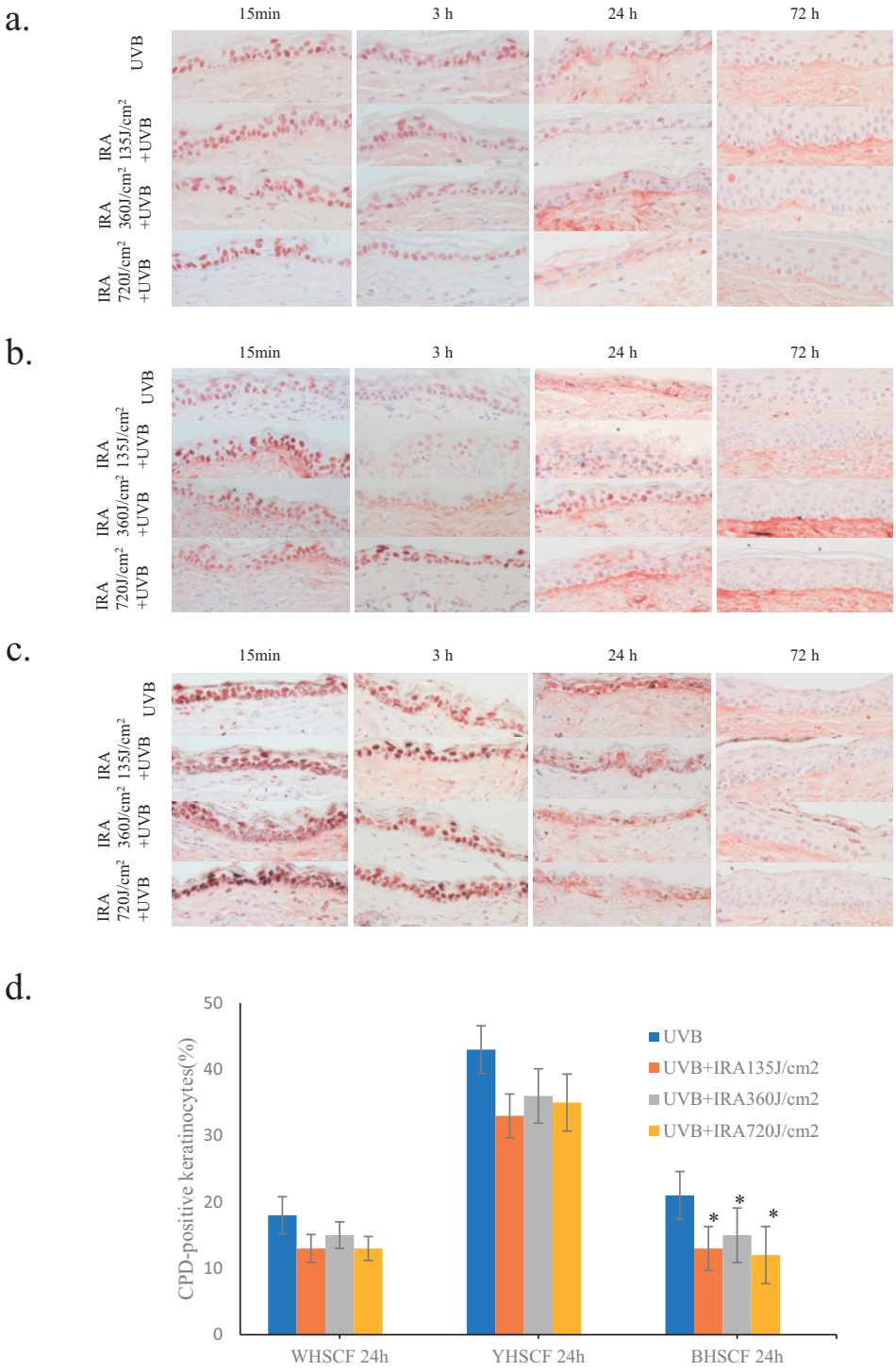

Fig. 1 Effect of pre-irradiation with infrared radiation A (IRA) on ultraviolet B (UVB) -induced cyclobutane pyrimidine dimer (CPD) formation. IRA was irradiated 3 h prior to UVB irradiation on WHSCF/*Xpa* KO mice, YHSCF/*Xpa* KO mice, and BHSCF/*Xpa* KO mice at doses of 135, 360, and 720 J/cm², respectively. 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).

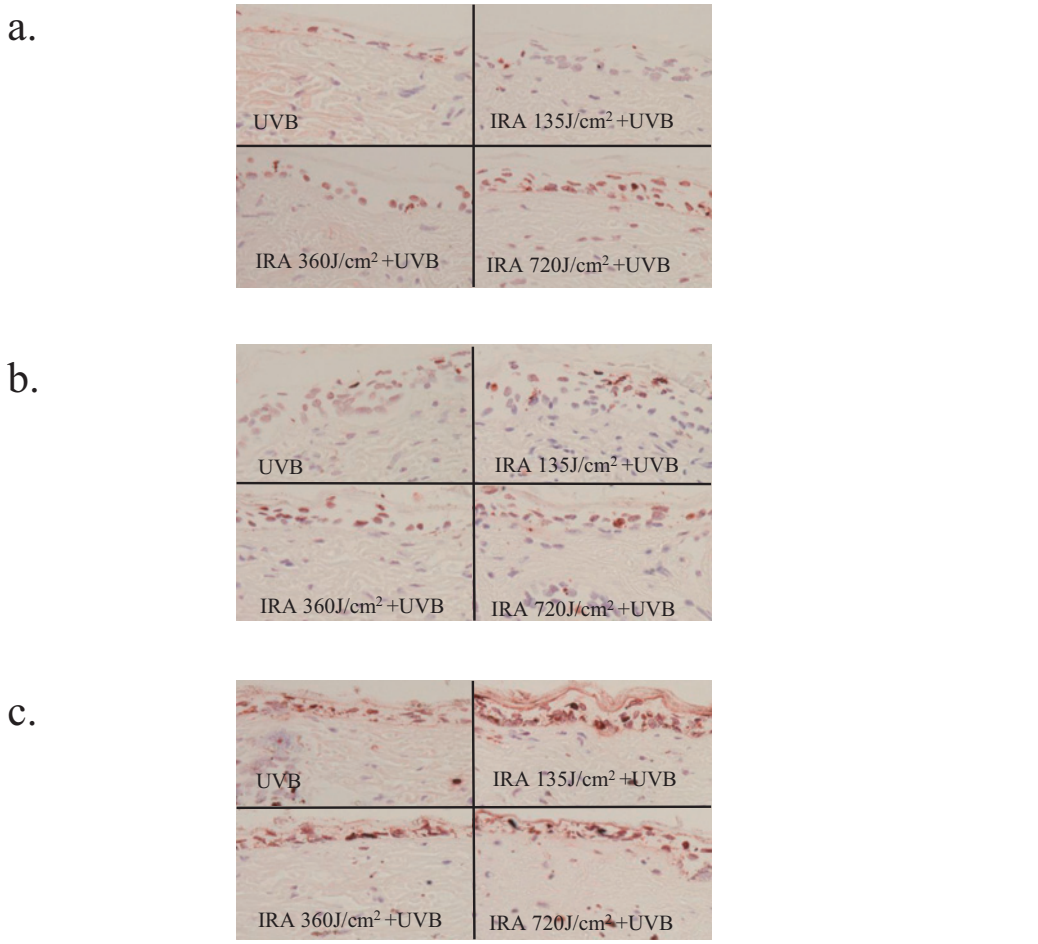

Fig. 2 Effect of pre-irradiation with infrared radiation A (IRA) on ultraviolet B (UVB) -induced apoptotic change. IRA and UVB were irradiated at doses similar to those shown in Figure 1. TUNEL assay was performed. (a) WHSCF/*Xpa* KO mice, (b) YHSCF/*Xpa* KO mice, and (c) BHSCF/*Xpa* KO mice. (d) Graph 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).

effect of IRA on melanin-burden skin, a detailed study on the pathway of enhancement of apoptotic changes by pre-irradiation with IRA might be needed.

Conflict of Interest: The authors declare no conflicts of interest.

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