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UVB-induced Damage and Photoreactivation in the Integument of the Terrestrial Isopod *Armadillidium vulgare*

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Summary——Recent studies have shown the molecular mechanisms of UV-induced damage such as the production of cyclobutane pyrimidine dimers (CPDs), increased apoptosis and DNA repair by photolyase. However, ultrastructural changes induced by UVB and subsequent mechanisms remain unsolved *in vivo,* due to the absence of suitable living animal models to investigate the photoreactivation following UV damage. In this study, we show that UVB-induced DNA damage and apoptosis are limited in the integument of woodlice *Armadillidium vulgare in vivo* and that the DNA damage is repaired by a photolyase excited by visible light. UVB (15.4 J/m^2, 2.4 kJ/m^2) radiation induces one type of DNA damage, CPD, in the integument of *A. vulgare*, and the number of apoptotic cells increased 9 hr after UVB exposure. Treatment with visible light accelerated the reduction of CPD and suppressed the increase in apoptosis induced by UVB. This study also provides evidence showing that UVB is blocked by two kinds of pigment cells, black and yellow chromatophores, and that clusters of pigment granules, which are usually distributed within chromatophores, are excreted into the epidermal cell layer beneath the cuticle. These results suggest that the photoreactivation mechanism and the pigment cell layer are important to protection from UVB in living *A. vulgare.*

Key words: UVB; DNA photoproduct; terrestrial isopod; ommochrome granule; apoptosis; cyclobutane pyrimidine dimers

1. INTRODUCTION

Ultraviolet B (UVB) radiation is one of the environmental factors that causes DNA

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damage within multicellular organisms. Cell death can be induced in physiological conditions after exposure to UVB. Genetically programmed cell death of various types of cells, called apoptosis, is a common phenomenon which includes internucleosomal cleavage of DNA and morphological changes (Hengartner, 2000). The morphological features of apoptosis include nuclear shrinkage and nucleoplasmic condensation which are followed by cytoplasmic condensation and fragmentation (Miller et al., 2002). The DNA fragmentation which accompanies apoptosis can be histochemically detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. In multicellular organisms, cells which have been irreversibly damaged by environmental stress undergo apoptosis and are actively removed (Janicke et al., 1998). Studies on the influence of UV radiation in various species revealed that UV exposure is harmful to basic biological phenomena in multicellular organisms such as normal development or morphogenesis of larvae (Baden et al., 1996; Soni and Joshi, 1997; Hofer and Mokri, 2000; Smith et al., 2000; Koponen and Kukkonen, 2002). Many studies have characterized the harmful effects of UV in invertebrates, most of them utilizing cultured cells. In the present study, we studied the structural changes of the integument caused by UV radiation of *Armadillidium vulgare*, a terrestrial isopod. Although sensitivity to UV radiation of multicellular organisms may differ in species or in various cell types, the system to repair damage caused by UV radiation seems to be common among multicellular organisms. *A. vulgare* has an integument structure similar to lower vertebrates, i.e. pigment cells are located under the epidermal cell layer (Negishi et al., 1994), while those cells are not differentiated in insects. This particular structure of the integument of *A. vulgare* is useful to examine the influence of UV on living multicellular organisms.

In vertebrates, the translocation of melanosomes after exposure to UV occurs in human skin (Jimbow and Fitzpatrick, 1975; Abdel-Malek, 1998) by a process in which melanosomes containing melanin are transferred from melanocytes to surrounding keratinocytes after UV exposure. The presence of a similar mechanism in invertebrates has been unknown. If the degradation of pigment granules occurs during the apoptosis of pigment cells, the elimination or destruction of pigment granules may be observed in the integument, a phenomenon that would provide important information about the protection mechanism against UV radiation of muticellular organisms. One of the aims in this study was to observe whether the transfer of pigment granules from the pigment cell layer to the epidermal cell layer occurs in the integument of *A. vulgare*.

UV radiation results in the generation of cyclobutane pyrimidine dimers (CPD) in DNA which can be repaired by specific enzymes, such as excision nucleases in the dark and/

or photoreactivating enzymes (DNA photolyases) in the light (Small and Greimann, 1977; Sancar, 1990; Sancar, 1994). Photorepair by DNA photolyase only occurs during exposure of cells to light in the visible wavelength range. Therefore, a visible light lamp was utilized to induce photoreactivation. No previous study on DNA repair has been carried out for photorepair in terrestrial isopods. Another aim of this study, therefore, was to clarify whether DNA repair systems are functioning in *A. vulgare*.

2. MATERIALS AND METHODS

2.1 UVB radiation

A. vulgare (red phenotype) were maintained at 20°C. For determination of CPD formation and/or electron microscopy, eight to ten adult isopods with body lengths of 5-10 mm were irradiated with a UVB lamp (FL15 TS, Toshiba, Tokyo, Japan) (exposure rate was 15.4 J/m^2, 290-320 nm) for 10 to 40 min as noted in the text. For immunohistochemistry of CPD and/or the TUNEL method, specimens were exposed to a dose 2.4 kJ/m^2 (30 min). Non-irradiated individuals of the same size were utilized as untreated controls.

2.2 Determination of DNA photoproducts

This method was carried out with a slight modification of the procedure described by Bennett et al. (2001). All experiments were performed under yellow light to avoid photoreactivation. Unirradiated or UVB-exposed specimens (immersed in liquid nitrogen (LN_2) after irradiation) were ground lightly in a chilled mortor for 45 s to reduce the specimens to fine powder. After the LN_2 evaporated, the powdered materials were transferred using a LN_2-chilled spatula to a droplet of Lysis buffer 1 [10 mM Tris-HCl, pH 8, 0.83 M EDTA, 2% (W/V) sarcosyl, 13% (W/V) mannitol, 1 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN)] in a plastic dish and buffer spreading was minimized. Each dish was then placed immediately in a desiccator, and was kept under a vacuum for 1 min.

The dissociated material was added to an equal volume of agarose solution [2% (W/V) SeaPlaque agarose (FMC, Rockland, ME, USA) in 10 mM Tris-HCl, pH 8, 1 mM EDTA, boiled for 10 min and held at 95°C], and the suspensions were pipetted into prechilled molds [GeNunc Modules (Nalge Nunc, cat. No 2-32549)] and placed on ice. After the plugs solidified, they were placed in 2 ml Lysis Buffer 2 [10 mM Tris-HCl, pH 8, 0.5 M EDTA, 2% sarcosyl, 1 mg/ml proteinase K] and digested at 42°C for 24 h without shaking. They were then replaced with 2 ml of the same solution and the digestion was

continued for 24 to 48 h. Plugs were rinsed three times with TE (10 mM Tris, pH 7.5, 1 mM EDTA), soaked in 4 ml phenylmethylsulfonate (PMSF) and then rinsed two to three times with TE.

CPD levels were determined as previously described (Quaite et al., 1992; Quaite et al., 1994). In brief, plugs in UV endonuclease buffer (30 mM Tris-HCl, pH 7.6, 1 mM EDTA, 40 mM NaCl) containing 1 μg/ml bovine serum albumin were digested with sufficient dimer-specific UV endonuclease to cleave all dimers while a companion plug was incubated in buffer alone. Enzyme activity and specificity toward CPDs were determined using supercoiled DNA containing or lacking CPDs. Plugs were rinsed two times with 2 mM EDTA, 30 mM NaOH, and then were incubated in 0.5 M NaOH, 50% (V/V) glycerol, 0.25% (W/V) bromocresol green for 30 min at 37℃. Samples along with molecular standards were electrophoresed in 0.4% alkaline agarose gels using unidirectional pulsed field gel electrophoresis. Gels were neutralized, stained with ethidium bromide (1 μg/ml in H_2O), and a quantitative electronic image was obtained with a charged coupled device-based camera-based system (AE-6901-DNAtype, ATTO, Tokyo, Japan). A DNA dispersion curve was calculated from the migration distance and molecular lengths of the DNA size standards; from a dispersion curve, and lane profiles of sample DNAs, the average molecular lengths of each + and − endonuclease companion pair were calculated, and from them, the lesion frequency in that sample was calculated, as previously reported (Freeman et al., 1986).

2.3 Immunohistchemistry and the TUNEL method

After UV irradiation, individuals were kept in the dark or the light for 0, 3, 6, 9, 12 and 24 hr, after which the specimens were fixed for 30 min in 4% paraformaldehyde (PFA) in PBS (pH 7.2) followed with three rinses in PBS. After the integument was dissected from the fourth and fifth segments of the dorsal region, the pieces were mounted in OCT Compound. CPDs were detected immunohistochemically using the monoclonal antibody TDM 20, which was generously provided by Dr. Matsunaga Tsukasa at Kanazawa University. Cryosections were fixed with 4% PFA for 15 min at room temperature, then treated with cold methanol (-20℃) for 30 min after washing with PBS containing 0.1% Tween 20 (PBST). The antibody was diluted 250-fold in PBST containing 5% fetal bovine serum (FBS) for 1 hr and then was visualized by Alexa Fluor 594 goat anti-mouse IgG (H+L) (Molecular Probes, USA) as a second antibody at 3 μg/ml in PBST containing 5% FBS for 30 min.

DNA fragmentation following UV irradiation was assayed by the TUNEL method. TUNEL staining was assessed using the “In situ cell death detection kit, fluorescein”

(Roche, Penzberg, USA) according to the manufacturer's instructions with slight modifications. The reaction mixture was diluted 80% with distilled H_2O containing 30 mM Tris-HCl (pH 7), 140 mM sodium cacodylate and 1 mM cobalt chloride, and all washing steps were performed using PBS containing 0.1% Tween 20 (PBST). Counterstaining was done with 1 µg/ml propidium iodide,10 µg/ml RNase in PBS. Samples were observed using fluorescence microscopy (ECLIPSE 80i, Nikon Co., Japan) or confocal laser scanning microscopy (FLUOVIEW FV 300, Olympus Optical Co. Ltd, Japan) and images were processed using Adobe Photoshop CS3 (Adobe Systems Incorporated).

2.4 Electron Microscopy

Integument pieces were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) overnight at 4℃, and were postfixed in 1% osmium tetroxide in sodium cacodylate buffer. The samples were then dehydrated through a graded ethanol series, embedded in epoxy resin, and cut with a Porter-Blum MT-2 ultramicrotome. The thin sections thus obtained were stained with uranyl acetate and lead citrate and were viewed with a JEOL 1001 electron microscope (JEOL Ltd. Japan) at 80 kV.

3. RESULTS

3.1 DNA damage and apoptosis induced by UVB

DNA damage induced by UVB was detected in *A. vulgare* by measuring amounts of CPD in the integument at various times after UVB exposure. CPD levels gradually increased when *A. vulgare* was exposed to UVB over 40 min (Fig. 1). Although the rate of CPD production varied among specimens, the mean value of CPD levels increased with exposure time to UVB. To determine the tissue specificity of CPD formation, frozen sections were examined by immunolabeling using an anti-CPD antibody (Fig. 2A). Virtually no CPD signals were detected in the non UVB-irradiated control. Positive signals for CPD were scattered over the integument which lies under the cuticle after just 30 min of UVB exposure although no signals were detected in the subcutaneous tissue. The TUNEL labeling assay was performed both in UVB-irradiated and in unirradiated *A. vulgare* to identify DNA fragmentation, a hallmark of apoptotic cell death (Fig. 2B). In contrast to the UVB-unirradiated *A. vulgare*, large numbers of TUNEL-positive signals were discernable in the integument 6 hr after 30 min of UVB-radiation. There were fewer signals determined in other regions of the sections. These results show that UVB damage is restricted to the integument.

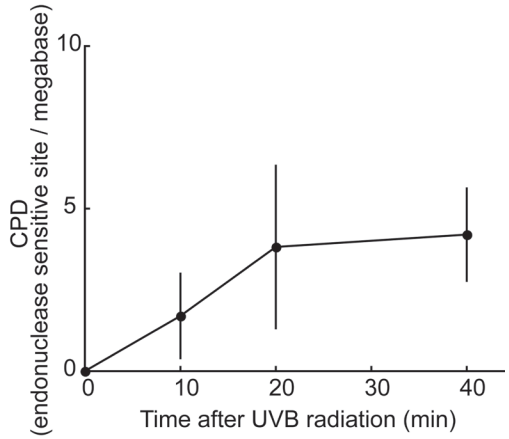

Fig.1 The induction of CPD in UVB-irradiated *A. vulgare* integument. A. *vulgare* was irradiated with UVB and kept in the dark. The initial slopes of the DNA repair time courses were determined from individual experiments. The level of CPD gradually increased according to the UVB exposure time.

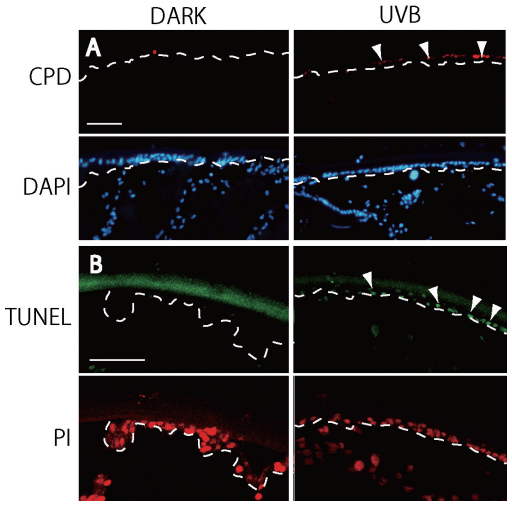

Fig.2 Appearance of CPD and apoptotic cells in the integument after UVB irradiation. (A) Frozen sections were subjected to immunohistochemical staining using an anti-CPD antibody (red signals, arrowheads) and were counterstained with DAPI (blue). *Left:* Untreated control which was not exposed to UVB showed no immunopositive cells. *Right:* CPD are detected in the epithelium (ep) but not in the subcutaneous tissues. Broken lines show the location of the basement membrane in Section of UVB irradiated specimen. (B) Appearance of apoptotic cells in the integument after UVB irradiation. TUNEL-stained (green signals arrowheads) and PI counter-stained (red signals) sections through the epithelium of *A. vulgare*. *Left:* Untreated control which was unirradiated showed fewer TUNEL-positive cells. *Right:* Section of UVB irradiated specimen; Fragmented DNA is detected in the epithelium but not in the subcutaneous tissues. Broken lines show the location of the basement membrane. Bar, 20 μm.

3.2 TEM analysis of morphological changes of cells in UVB-irradiated integument

Figure 3A shows a transverse-section of the dorsal integument of *A. vulgare*, which consists of epidermal cells and two kinds of pigment cells, ommochrome chromatophores and yellow-colored chromatophores. The cuticles were developed in the external regions of the epidermis, whereas the basal lamina lies under the pigment cells. No cells revealing any morphological features of apoptosis were found in the non UVB-irradiated controls. Both epithelial cells and pigment cells had a homogenous appearance of their nuclear material.

Both epidermal cells and pigment cells in the integument 10 hr after exposure to UVB for 30 min frequently showed characteristic changes in the clumping of nuclear chromatin at the periphery of the nuclei (Figs. 3B-3D). The nuclear membranes had numerous detached ribosomes and had often shrunken at one corner. Sometimes the nuclear membranes appeared in small fragments. These features were characteristic of apoptosis caused by UVB radiation. Changes in the localization of pigment granules were often found in the integument after UVB-exposure. Usually, pigment granules are not found in epidermal cells since pigment cells in *A. vulgare* are distinguished from epidermal cells beneath the cuticle. However small clusters of pigment granules were frequently observed in the epidermal cell layer after exposure to UVB (Fig. 3E). Pigment granules enclosed by a membrane looked like protrusions of pigment cell dendrites. A number of 10 nm filaments were distributed along pigment granules within the protrusions, and microtubules were associated outside the organelles (Fig. 3F).

3.3 Impact of visible light on the UVB-irradiated integument of *A. vulgare*

To identify the photoreactivation of UVB damage, we quantitatively analysed CPD formation in the epithelium of *A. vulgare* which was treated with visible light after UVB radiation (Fig. 4A). UVB exposure caused CPD damage in 38% of epidermal cells. Visible light significantly decreased the number of CPD-positive cells to 19% after 30 min of UVB radiation (Fig. 4A; Light). On the other hand, the percentage of CPD-positive cells was unchanged in the visible light untreated group (Fig. 4A; Dark) after 30 min. At 3 hr after UVB radiation, there was no significant difference between the visible light treated group and the untreated group in the percentage of CPD-positive cells. We also investigated the impact of visible-light on apoptosis using the TUNEL method (Fig. 4B). In the visible light untreated group, some cells had begun apoptosis within 3 hr after UVB radiation. The largest numbers of apoptotic cells were found after 6 hr (34.1%) to 9 hr (44.2%) of UVB radiation. The visible light decreased the number of apoptotic cells. The proportion of apoptotic cells in visible light treated *A. vulgare* was

Fig.3 Electron micrograph of the integument of unirradiated (A) and UVB radiated (B)-(F) *A. vulgare*. (A) Epidermal cells (ep) under the cuticle (c) cover the ommochrome chromatophore (oc). (B)-(F) Epidermis (B), ommochrome chromatophore (C) and yellow-colored chromatophore (D) are undergoing apoptosis and nuclear chromatin has accumulated at 10 h after UVB radiation. Arrowheads show where the nuclear membrane has detached from the periphery of nuclei. (E) Clusters of pigment granules (arrowheads) transported from the ommochrome chromatophore (oc) to the epidermal cell layer (ep) under the cuticle (c), which is an unusual phenomenon observed in the integument after UVB exposure for 30 min. (F) A number of filaments (arrowheads) are observed along with pigment granules transferred into the epidermal cell layer, and microtubules (arrow) are seen near the pigment granules. Bars, 2 µm in (A) and (E) and 500 nm in (B)-(D) and (E).

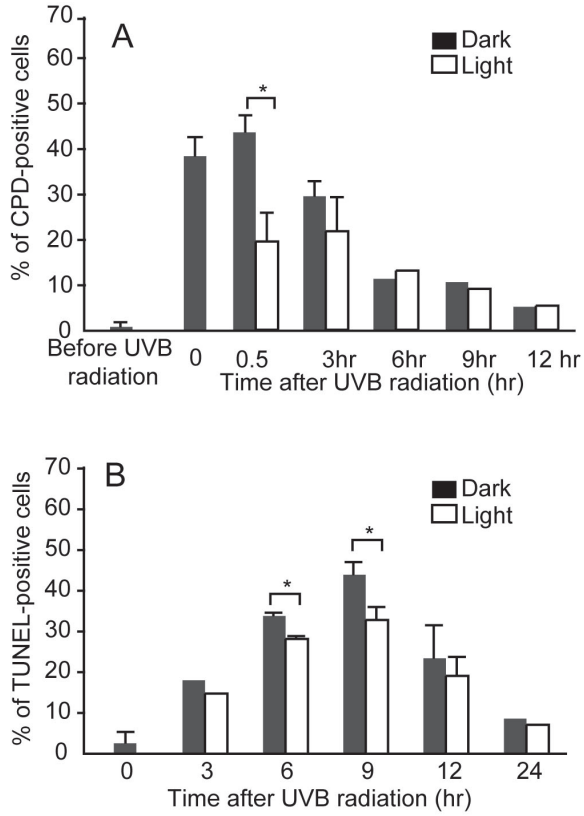

Fig.4 Photoreactivation in *A. vulgare* integument. The ratio of CPD-positive cells (A) and TUNEL-positive cells (B) in *A. vulgare* integument with (Light) or without (Dark) visible light exposure after UVB radiation. CPD levels were decreased in the visible light treated group 30 min after UVB radiation (A). At each time after UVB radiation, the visible light treated group had fewer apoptotic cells than the untreated group (B). (*P<0.05, Student's t-test,).

15% lower than in untreated *A. vulgare* after 9 hr. Thus, photoreactivation functions *in vivo* in *A. vulgare*.

4. DISCUSSION

In vertebrates, a number of studies have shown the effects of UV such as damage to DNA or the plasma membrane and the photorepair induced by visible light in integument cells. However, most of those studies were carried out using cultured cells or in transgenic mice expressing photolyase. In the present study, we utilized living terrestrial isopods to observe the effects of UVB and the impact of visible light on the integument. We found morphological changes and DNA damage produced by UVB

radiation and the photoreactivation by visible light in cells of *A. vulgare* integument. When *A. vulgare* was exposed to 2.4 kJ/m^2 UVB for 30 min, the isopod lived for more than one week at room temperature. Therefore, the morphological changes mentioned above may have not resulted in lethal damage to the isopod due to the UVB radiation.

CPD formation and the induction of apoptosis were restricted in the integument (Fig. 2). Each component of the integument, i.e. the epidermis and the two kinds of chromatophores, exhibited features of apoptosis which include characteristic nuclear chromatin condensation (Fig. 3). In many animals, it has been reported that UVB is blocked by black pigment cells, termed melanophores. It has been suggested that in medaka, colored pteridines of yellow chomatophores and xanthophores, also protect the tissues from UV (Armstrong et al., 2000). The yellow-colored chromatophores in *A. vulgare* contain pteridine-containing granules (Negishi et al., 1998). Thus, the two kinds of chromatophores in *A. vulgare* seem to collaborate to protect the underlying tissues of the skin, such as the mesenchyme, nerves and muscles, from UVB-induced damage.

The removal of the nuclear membrane from apoptotic cells is frequently observed in integument cells after UVB radiation (Fig. 3B). The remarkable feature is that a large number of ribosomes aggregate along the nuclear membrane, which suggests that digestion following withdrawal of membranous components takes place in epidermal cells undergoing apoptosis. Cell disassembly during apoptosis by caspases and/or by other enzymes are ubiquitous phenomena in living organisms (Thornberry and Lazebnik, 1998), however, changes in the ultrastructure of apoptotic cells during disassembly have been slightly revealed (Faleiro and Lazebnik, 2000). The most remarkable change in cells during apoptosis is recognized to be the disassembly of nuclear membranes, because soluble substrates must be located in the cytoplasm or nucleus without any barrier. Therefore, the removal of the nuclear membrane observed in this study may help accelerate apoptosis following the generation of nuclear pores. Whether removal of the nuclear membrane is common in apoptosis of invertebrates is not yet known.

The present study also provides the first evidence that, in the integument of invertebrates having ommochromes as a pigment, pigment granules translocate from pigment cells to epidermal cells through extensions of pigment cell dendrites after UVB exposure (Fig. 3D). The translocation of pigment granules and 10 nm filaments distributed along them (Fig. 3E) that was observed in *A. vulgare* integument after UVB exposure resembled the phenomenon induced in human cells in which melanosomes are transferred from melanocytes to keratinocytes following exposure to UV (Jimbow and Fitzpatrick, 1975; Jimbow and Sugiyama, 1998). This melanosome translocation was thought to help reduce the amount of UV damage, however, recently the increased

melanin content following exposure to UV was suggested to be a consequence of DNA repair (Eller et al., 1994). Therefore, in the case of *A. vulgare,* the translocation of pigment granules after UVB exposure may also be induced as the result of DNA repair.

In the present study, we showed that 30 min of visible light treatment dramatically decreased CPD levels induced by UVB radiation. The number of apoptotic cells in the visible light treated integument was 60% less than in untreated controls 9 h after the UVB exposure. Therefore, this suggests that visible light exposure probably activates photolyases to repair DNA photoproducts such as CPDs and that DNA photoproducts trigger cell apoptosis in the integument of *A. vulgare.*

Whether the major DNA photoproduct induced by UVB radiation in *A. vulgare* is CPD, as in mammalian cells (Mitchell, 1988) has not been determined in this study. In invertebrates, DNA damage induced by UV radiation has not been previously reported, however, the present results demonstrate the similarity of DNA photoproducts between vertebrates and invertebrates caused by UV, which is consistent with the evolution of DNA repair mechanisms in animals on the earth. In the present study, it is clear that the number of apoptotic cells are greater than the CPD-positive cells. In vertebrates and invertebrates, 6-4 photoproducts are known as another type of DNA damage. It might be that not only CPD but also other photolesions such as 6-4 photoproducts are formed by UVB in *A. vulgare* integument.

These results suggest that *A. vulgare* has a distinct protective response to UVB exposure and is suitable as a model for the investigation of effects of UV in higher vertebrates, which are very difficult to study.

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