INDUCTION OF UNSCHEDULED DNA SYNTHESIS IN HAIRLESS MOUSE EPIDERMIS BY ULTRAVIOLET LIGHT

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ABSTRACT — The activity of ultraviolet (UV) light to induce unscheduled DNA synthesis (UDS) was investigated in hairless mouse epidermis by means of an in vivo - in vitro assay using a liquid scintillation counting method. Groups of three to five 8-week-old female hairless mice were irradiated with UV-B or UV-A, then skin samples were taken and cultured individually in medium containing [3H]thymidine with or without hydroxyurea (HU) for 2 h. DNA of the epidermis was extracted, and incorporation of [3H]thymidine and the DNA content were determined with a liquid scintillation counter and a fluorescence spectrophotometer, respectively. Induction of UDS was judged in terms of the UDS index [(the ratio of DNA synthesis in the presence of HU to that in its absence)×100]. UV-B increased the UDS index 1 hr after irradiation of 500 J/m2, which corresponds to approximately 1 minimal erythema dose or 1 minimal edema dose, and showed a dose-dependent increase up to 17-fold in the UDS index at irradiation doses of 500 to 2,000 J/m2. In a time-course study, UV-B also increased replicative DNA synthesis (RDS) 48 hr after irradiation at 1,000 J/m2. On the other hand, UV-A did not increase the UDS index at irradiation doses of 2×103 to 8×105 J/m2. These results show that induction of UDS by UV irradiation depends on wavelength, and an increase of RDS in the epidermis exposed to UV-B irradiation appears after induction of UDS.

KEY WORDS: Unscheduled DNA synthesis, Replicative DNA synthesis, Hairless mouse epidermis, Ultraviolet light, Minimal erythema dose, Minimal edema dose

INTRODUCTION

The ultraviolet (UV) component of sunlight is known to have various acute and chronic effects on biological systems. Carcinogenesis in the skin is one of the most important chronic effects. UV light is subdivided into three parts, i.e., UV-A (315-380 nm), UV-B (280-315 nm) and UV-C (100-280 nm) (Kelfkens et al., 1991). UV-C is carcinogenic (Lill, 1983), but is filtered out by atmospheric ozone, and does not reach the surface of the earth (Taylor et al., 1990). On the other hand, UV-A and UV-B can reach the surface of the earth. UV-B causes sunburn and suntan as acute biological responses. UV-A scarcely contributes to sunburn, though excessive UV-A brings about suntan. Carcinogenicity of UV-B has been established (Freeman, 1975; Bissett et al., 1989). Some investigators found no effect of UV-A (Staberg et al., 1983; Kaase et al., 1984), but it is now established as a carcinogen as well (Sterenberg et al., 1990; van Weelden et al., 1990; Kelfkens et al., 1991), though it is far less potent than UV-B (approximately 1,000 times less) (Slominski and Pawelek, 1998). Unscheduled DNA synthesis (UDS) reflects the

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genotoxicity of carcinogens. Many investigators have observed induction of UDS by UV in cultured epidermal or dermal cells in vitro (Hosomi and Kuroki, 1985; Machino et al., 1986; Kondo et al., 1987) and in the skin in vivo, using autoradiographic methods (Bishop, 1979; Ishikawa et al., 1984; Machino et al., 1986; Höögsmann et al., 1987; Potten et al., 1993). In a recent study, we detected induction of UDS in hairless mouse epidermis (Mori et al., 1999) by modifying an in vivo - in vitro assay using the liquid scintillation counting method which was developed for the glandular stomach, forestomach, colon and liver of rats (Furihata et al., 1984a; 1985; Furihata and Matsushima, 1987a; Sawada et al., 1989; Ohsawa et al., 1993; Furihata and Matsushima, 1995) as a faster and easier method than autoradiography. We established that the UDS index was a valid predictor of carcinogenicity of chemicals in the skin (Mori et al., 1999). The in vivo - in vitro assay using liquid scintillation counting is also reported to be able to detect not only potential tumor-initiating activity in terms of induction of UDS, but also potential tumor-promoting activity in terms of an increase of replicative DNA synthesis (RDS) in simultaneous cultures with and without hydroxyurea (HU) (Furihata et al., 1985; Furihata and Matsushima, 1995), which is an inhibitor of RDS (Adams and Lindsay, 1967). In the present study, we investigated induction of UDS by UV-B and UV-A in hairless mouse epidermis using our method. We also tried to detect the potential tumor-promoting activity of UV-B by observing the change of RDS in a time-course study. The minimal erythema dose (MED) and the minimal edema dose (MED) of UV-B irradiation in hairless mice were determined, and were utilized in deciding the dose range in these assays. Furthermore, we compared the induction patterns of erythema, edema, RDS and UDS in time-course studies after UV-B irradiation to see whether there is any relationship among them.

**MATERIALS AND METHODS**

**Chemicals**

[CH$_3$-H]$^3$H]Thymidine ([$^3$H]dThd, 2.37 - 2.90 TBq/mnmole) was obtained from ICN Radiochemicals (Irvine, CA).

**Ultraviolet radiation sources**

For UV-B, seven fluorescent tubes, which emit at 270-380 nm (FL20S -E-30/DMR lamp, Toshiba Light and Technology, Tokyo), were used. The output intensity was 0.456 mW/cm$^2$. For UV-A, fourteen fluorescent tubes, which emit at 300-440 nm (FL32S-BL/DMR lamp, Toshiba Light and Technology, Tokyo), were used. The incident light was passed through a 3-mm-thick ultraviolet absorbing glass filter (Longpass filter WG 335, Schott, Mainz, Germany) to eliminate wavelengths below 320 nm. The output intensity was 4.92 mW/cm$^2$. These fluorescent tubes were fitted on a medical ultraviolet irradiation apparatus ( Dermaray M-DMR-100, Ei Sai Co., Tokyo), and ultraviolet intensities were measured using a UV-radiometer ( UVR-305/365-D, Ei Sai Co., Tokyo).

**Animals and treatments**

Female 8-week-old hairless mice (HOS:HR-1, Hoshino Co., Saitama, Japan) were allowed food and water ad libitum until termination of the experiment and were housed in an air-conditioned room with a 12 hr light/12 hr dark cycle. Mice were placed under the medical ultraviolet irradiation apparatus, and were irradiated with UV-B or UV-A, 500 J/m$^2$ of UV-B, which corresponds to approximately 1 MED or 1 MEdD, was adopted as the minimum dose in the dose-response study of UV-B. Since the biological effects of UV-A are much lower than that of UV-B (Slominski and Pawelek, 1998), 2 × 10$^3$ J/m$^2$ of UV-A, which is a more than sufficient dose, was adopted as the minimum dose in the dose-response study of UV-A. Animal experiments in our laboratory are conducted in accordance with the guidelines for animal experiments of the National Institute of Health. The experimental protocol of this study was approved by the institutional review board of the Shiseido Research Center for Animal Welfare.

**Preparation of epidermal extracts**

Preparation of epidermal extracts was performed as described previously (Mori et al., 1999). Mice underwent euthanasia at the indicated times after irradiation. Two skin pieces of approximately 177 mm$^2$ from the skin that had been irradiated with UV-B or UV-A were taken and the subcutis was removed. Each portion was cut into halves, and halves from the upper back portion and the lower back portion were paired. These samples were cultured in 3 ml of L-15 medium containing 100 µg of streptomycin/ml, 100 units of penicillin G/ml and 370 kBu [H]$^3$H]dThd/ml with or without 10 mM HU, at 37 °C for 2 hr in L-shaped glass tubes with gentle shaking (60 oscillations/min). The tissue was washed with 3 ml of L-15 medium containing 100 µg of streptomycin/ml, 100 units of penicillin G/ml, and then incubated at 37 °C for 30 min in 3 ml of phos-
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phate-buffered saline containing 1 mM unlabeled dThd, and 500 mM ammonium thiocyanate (Hatao et al., 1993) to isolate the epidermis from each sample. The sample was then washed with 3 ml of phosphate-buffered saline, and the epidermis was separated with tweezers. The epidermis was homogenized in 1 ml of 0.5% sodium dodecylsulfate containing 300 mM sodium chloride and 30 mM tetraborate ethylenediaminetetraacetate. The homogenate was acidified with 150 µl of 70% trichloroacetic acid, and the precipitate was washed with 1 ml of 10% trichloroacetic acid, homogenized in 1 ml of 1 N potassium hydroxide, and incubated at 37°C overnight to hydrolyze RNA. On the next day, the supernatant was neutralized with 200 µl of 5 N hydrochloric acid, and DNA and protein were precipitated with 60 µl of 70% trichloroacetic acid. The precipitate was homogenized in 700 µl of 5% trichloroacetic acid and incubated at 80°C for 30 min to hydrolyze DNA. The DNA fraction was obtained as the supernatant after centrifugation at 3,000 rpm for 10 min.

**Measurements of DNA synthesis**

DNA synthesis was measured as described previously (Mori et al., 1999). Aliquots of 500 µl of the supernatant with HU and 100 µl of that without HU were dissolved in 3 ml and 1 ml of ACS II scintillant (Amersham Corp., Arlington Heights, IL), respectively, and incorporation of [3H]dThd into DNA was determined with a liquid scintillation counter. The DNA content of the supernatant was determined by using a fluorescence spectrophotometer as described previously (Furuhata et al., 1985). Aliquots of 50 µl of the supernatant were incubated with 100 µl of 2 M 3.5-diaminobenzoxic acid dihydrochloride (Tokyo Kasei Kogyo Co., Tokyo, Japan) at 37°C for 30 min and 600 µl of 0.6 N perchorlic acid was added. The fluorescence of the mixture was then determined at excitation and emission wavelengths of 410 nm and 510 nm, respectively. Calf thymus DNA (Sigma Chemical Co., St. Louis, MO) was used as a standard.

**Calculations of the UDS index**

The UDS index was calculated as described previously (Mori et al., 1999). It was expressed as the ratio (×100) of the amount of DNA synthesis in the presence of HU to that in its absence.

\[
\text{UDS index} = \frac{[\text{DS}(+\text{HU})]/[\text{DS}(-\text{HU})]}{100}
\]

[DS(+HU)] and [DS(-HU)] mean the observed DNA synthesis in the presence and in the absence of HU, respectively.

**Determination of MEdD and MEdD**

The MEdD and MEdD of UV-B irradiation in hairless mice were determined as described previously (Freeman et al., 1988). The back of the hairless mouse was exposed to graduated doses of UV-B in 25% increments at four circles 9 mm in diameter. The range of irradiation doses was from 400 to 781.25 J/m². At 48 hr later, erythema and edema on the exposed sites were observed.

**Assessment of erythema and edema**

Erythema and edema after UV-B irradiation were assessed as described previously (Sato et al., 1981; Kato et al., 1985). Each reaction was scored according to the following criteria: for erythema formation, 0, no erythema; 1, very slight erythema; 2, well-defined erythema; 3, moderate to severe erythema; 4, severe erythema, and for edema formation, 0, no edema; 1, slight edema; 2, moderate edema; 3, severe edema.

**Statistical analysis**

All data were expressed as the mean ± standard error (S.E.). Statistical significance was determined by the use of Student’s t (Welch) test (Gad and Weil, 1982).

**RESULTS**

**MEdD and MEdD**

The MEdD and MEdD of a group of four mice were 562.50 ± 36.08 and 506.25 ± 46.07 J/m², respectively. These results were utilized to set the minimum dose of UV-B in the dose-response study.

**Induction of UDS by UV-B**

In the dose-response study, DNA synthesis in the presence of HU was increased at 1 hr after 500, 1,000 and 2,000 J/m² UV-B irradiation, while DNA synthesis in the absence of HU showed a dose-dependent decrease (Fig. 1A). The UDS index was increased over this dose range, showing an approximately 17-fold increase at 2,000 J/m² UV-B (Fig. 1B). In the time-course study, DNA synthesis in the presence of HU was significantly increased at 1 hr after 1,000 J/m² UV-B (Fig. 2A), and DNA synthesis in its absence was decreased at 1, 2, 4 and 8 hr, but was significantly increased at 48 hr (Fig. 2B). The UDS index was at the maximum level at 1 hr (Fig. 2C). Thus, the induction of UDS was accompa-
nied with a decrease of RDS in the early stage, followed by an increase of RDS at 48 hr. UV-B showed potential tumor-initiating activity (UDS) in the early stage, but potential tumor-promoting activity (RDS) at 48 hr in hairless mouse epidermis.

Non-induction of UDS by UV-A

Since UV-B showed the maximum level of induction of UDS at 1 hr in the time-course study, the dose-response study of UV-A was carried out at 1 hr. DNA synthesis both in the presence and absence of HU was unchanged at 1 hr after $2 \times 10^5$, $4 \times 10^5$ and $8 \times 10^5$ J/m² UV-A irradiation (Fig. 3A). The UDS index was unchanged over this irradiation range (Fig. 3B). The dose of $8 \times 10^5$ J/m² of UV-A was 1,600 times the minimum irradiation dose of UV-B which caused induction of UDS. Thus, the ability of UV-A to induce UDS was exceedingly low in comparison with that of UV-B under our experimental conditions.

Time-dependent induction of erythema and edema by UV-B

The pattern of erythema produced after 1,000 J/m² UV-B was biphasic (Fig. 4A). Erythema first appeared at 2 hr, and reached the maximum level at 4 hr. This reaction disappeared completely at 12 hr. The second erythema appeared at 24 hr, and reached the maximum at 48 hr, being accompanied by the maximum level of edema (Fig. 4B). The patterns of erythema and edema were compared with the patterns of DNA synthesis in the absence of HU and the UDS index (Fig. 4C and 4D), calculated as percentages of the control values (100%). The time of maximum DNA synthesis in the absence of HU (% of control) coincided with the time of the maxima of the second erythema and edema (Fig. 4A, 4B and 4C).

DISCUSSION

Recently, we reported that the method using liquid
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Scintillation counting was useful for detecting induction of UDS by chemicals in the skin (Mori et al., 1999). In that study, we incorporated a tape-stripping procedure for removal of the stratum corneum in order to enhance percutaneous absorption of the applied chemical. We omitted this procedure in this study, because UV-A and UV-B can penetrate the epidermis (Hruza and Pentland, 1993). Induction of UDS by UV-B was detectable at the irradiation dose of 500 J/m², which corresponded to approximately 1 MErd or 1 MErd, whereas induction of UDS by UV-A was undetectable even at a 1,600 times larger irradiation dose. Thus, the ability of UV-A to induce UDS seems to be exceedingly low in comparison with that of UV-B. UV-B is also a strong carcinogen (Freeman, 1975; Bissett et al., 1989), whereas UV-A is a weak carcinogen (Sterenberg et al., 1990; van Weelden et al., 1990; Kelfkens et al., 1991). Thus, non-induction of UDS by UV-A under our experimental conditions might account for the low genotoxicity.

We used HU, an inhibitor of ribonucleotide reductase (Elford, 1968), to inhibit RDS. Ribonucleotide reductase is responsible for controlling the deoxyriboonucleoside triphosphate pool size (Zhou et al., 1998). It is reported that inhibition of ribonucleotide reductase by HU reduces RDS but not UDS in various cells, including human skin (Katz and Sirover, 1987; Synder, 1984). The results suggest that fluctuations in the deoxynucleoside triphosphate pools do not limit the extent of excision-repair synthesis in cells and demonstrate that DNA nucleotide excision-repair synthesis does not significantly diminish the size of the deoxynucleoside triphosphate pool. Cases in which DNA synthesis in the presence of HU is increased and that in its absence is decreased are regarded as clear induction of UDS, while those in which DNA synthesis in the pres-

![Fig. 3. Effect of UV-A irradiation on DNA synthesis. The skins were obtained at 1 hr after irradiation of UV-A. Fig. A and B show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean±S.E. for groups of five mice. None of the irradiated groups showed a significant difference from the control group.](image)

![Fig. 4. Time-dependent induction of erythema and edema by UV-B and comparison with changes of DNA synthesis. For Fig. A and B, the skin reactions were observed at the indicated times after exposure to 1,000 J/m² UV-B. Fig. A and B show the scores of erythema and edema. Values are the mean±S.E. for groups of five mice. For Fig. C and D, the data were calculated as percentages of the control values (100%) for DNA synthesis in the absence of HU and the UDS index (see Fig. 2B and 2C, respectively). Values are the mean±S.E. for groups of three mice.](image)
ence of HU is unchanged and that in its absence is decreased are also regarded as indicating slight induction of UDS (Mori et al., 1999). Induction of UDS by 500 J/m² irradiation of UV-B was clear, with an increase of DNA synthesis in the presence of HU and a decrease in its absence. It is possible that UDS is induced by UV-B even at doses of less than 500 J/m², which do not induce erythema and edema.

In the time-course study, DNA synthesis in the absence of HU showed the maximum decrease at 1 to 2 hr and the maximum increase at 48 hr after UV-B irradiation. This pattern was similar to the pattern of DNA synthesis in the absence of HU in hairless mouse epidermis determined with autoradiography (Epstein et al., 1970). We reported previously that application of chemical carcinogens causes a reduction of RDS, and this reduction should be helpful for selecting optimal doses for the UDS assay (Mori et al., 1999). In the present study, UV-B induced UDS at irradiation doses which reduce RDS, and a decrease of RDS was shown to be a useful criterion for selecting doses of not only chemical carcinogens but also environmental physical carcinogens, such as UV-B. The decrease of RDS by UV-B might be caused by structural changes in DNA, such as formation of DNA photoproducts, which would interfere with the normal replication of DNA. On the other hand, an increase of DNA synthesis in the absence of HU is useful for detecting the tumor-promoting activity of chemicals in rat stomach (Furihata et al., 1984b), and was also available for hairless mouse epidermis. However, the increase of DNA synthesis in the absence of HU in hairless mouse epidermis by UV-B was not high and reached the maximum at 48 hr, in contrast to within 24 hr in rat glandular stomach after oral administration (Furihata et al., 1984b, 1985, 1987b; Furihata and Matsushima, 1995). The small increase of RDS might be related to the morphological features of the skin, i.e., the existence of the stratum corneum might disturb the increase of RDS. In addition, a decrease of RDS at an early stage seems to delay the increase of RDS at a later stage. The decrease of RDS signifies toxicity to cells, and it took 12-24 hr to recover from the reduction of RDS after irradiation (Fig. 2B). The increase of RDS in rat glandular stomach occurs without a decrease of RDS at an early time (Furihata et al., 1984b, 1985, 1987b; Furihata and Matsushima, 1995). The magnitude of the RDS decrease at an early stage might determine the time of RDS increase.

It has been reported that the pattern of erythema produced after UV-B irradiation might be biphasic (Hruza and Pentland, 1993). In the present study, the appearance of erythema after UV-B irradiation was biphasic, and the two phases seemed to be induced by different mechanisms. Young et al. (1996) reported that there was an inverse relationship between the onset of erythema and the repair of (6-4) photoproduct. The (6-4) photoproduct, one of the kinds of DNA damage observed in UV-irradiated DNA, is produced by linkage between the C⁶ position of one thymine and the C⁴ position of an adjacent thymine. They suggested that (6-4) photoproduct excision repair or the formation of fragments might initiate erythema. Yarosh and Kripke (1996) reported that DNA damage directly causes release of cytokines, which contribute to erythema reaction. Active oxygen species, such as the superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), singlet oxygen (O₁₂) and the hydroxyl radical (OH·), and chemical mediators, such as prostaglandins and histamine, are also generated or released by UV-B, and contribute to DNA damage and erythema reaction, respectively (Hruza and Pentland, 1993; Yarosh and Kripke, 1996).

In the second erythema reaction, prostaglandins seem not to make a direct contribution, because erythema persisting after 24 hr of UV-B irradiation is not suppressible by indomethacin (Hruza and Pentland, 1993). However, Eglestein and Weinstein (1975) reported that intradermal injection of prostaglandin E₂ produced an increase in the number of DNA-synthesizing cells at 48 hr, and they suggested that prostaglandin E₂ might be one of the chemical mediators of the UV-induced increase in DNA-synthesizing cells. We consider that these reactions might be caused by release of other chemical mediators induced by prostaglandins. In addition, cell proliferation mediated by active oxygen species (Murrell et al., 1990) would also tend to increase RDS. Several inflammatory cytokines, which cause induction of intercellular adhesion molecule-1 (ICAM-1) expression, presumably contribute to the production of erythema and edema. It is thought that UV-B immediately suppresses interferon-γ-induced ICAM-1 expression, which is restored in 24 hr and then results in an induction of ICAM-1 expression after 48 hr (Yarosh and Kripke, 1996). Thus, the coincidence of the times of the maximum levels of second erythema and edema appearance, and RDS increase might be interpreted as reflecting the interaction among cell proliferation mediated by active oxygen species, and release of chemical mediators and inflammatory cytokines.

Our results showed that UV-B induced marked UDS. We were also able to detect the potential tumor-promoting activity of UV-B. The method was thus
shown to be valid for detection of environmental physical skin carcinogens, as well as chemical skin carcinogens. Induction of UDS and increase of RDS in hairless mouse epidermis by UV-B irradiation seems to be related to the induction of erythema and edema, but further work will be necessary to elucidate the mechanisms involved.

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