INDUCTION OF UNSCHEDULED DNA SYNTHESIS IN HAIRLESS MOUSE EPIDERMIS BY 8-METHOXYPsorALEN PLUS ULTRAVIOLET A (PUVA)

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ABSTRACT Induction of unscheduled DNA synthesis (UDS) by 8-methoxypsoralen (8-MOP) plus ultraviolet A (UV-A) (PUVA) was investigated in the epidermis of female hairless mice 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 had 8-MOP applied once onto two areas of the back after stripping of the stratum corneum with adhesive tape to enhance skin penetration, and were irradiated with UV-A. Skin samples were taken and cultured in a medium containing [³H]thymidine with or without hydroxyurea (HU) for 2 hr. DNA of the epidermis was extracted, and the incorporation of [³H]thymidine into DNA 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]. In a time-course study, the UDS index was increased at 1, 2 and 24 hr after 1×10^5 J/m² UV-A irradiation with 0.001% 8-MOP, reaching the maximum level at 24 hr. In a dose-response study, it was significantly increased at the dose of 1×10^5 J/m² of UV-A at 24 hr with 0.001% 8-MOP, but showed no significant change at the doses of 0.5×10^5 , 2×10^5 and 4×10^5 J/m². In a further study on the effect of varying the dose of 8-MOP, the UDS index was significantly increased at 0.001 and 0.002% 8-MOP at 24 hr after 1×10^5 J/m² UV-A irradiation, reaching the maximum level with 0.002% 8-MOP. The increase of the UDS index in these studies was less than 3-fold. These results show that PUVA causes a small induction of UDS, which might be due to slow DNA excision repair over a long period.

KEY WORDS: Unscheduled DNA synthesis, Replicative DNA synthesis, Hairless mouse epidermis, 8-Methoxypsoralen, Ultraviolet light, PUVA

INTRODUCTION

A combination of 8-methoxypsoralen (8-MOP) and ultraviolet A (UV-A) radiation, commonly referred to as PUVA, is known to inhibit cell proliferation, and this inhibitory effect is widely utilized for the treatment of skin diseases, such as psoriasis, vitiligo and mycoses (Lerner *et al.*, 1953; Parrish *et al.*, 1974; Gilchrest *et*

al., 1976). Despite the widespread use, epidemiological studies have suggested that PUVA treatment might increase the risk of cutaneous squamous cell carcinoma in humans (Stern *et al.*, 1988; Lindelöf *et al.*, 1991). In rodents, carcinogenicity of PUVA to the skin has been established by oral administration (p.o.), intraperitoneal administration (i.p.) or dermal application of 8-MOP in combination with ultraviolet A radiation. (Griffin,

1959; Dubertret *et al.*, 1979; Hannuksela *et al.*, 1986). PUVA is also mutagenic, and can cause reverse mutations in bacteria (Dean *et al.*, 1991; Chételat *et al.*, 1993a) and yeast (Chételat *et al.*, 1993a), as well as chromosomal aberrations (Dean *et al.*, 1991; Chételat *et al.*, 1993b), micronucleus induction (Kersten *et al.*, 1999), sister chromatid exchanges (Loveday and Donahue, 1984) and gene mutations (Cristofolini *et al.*, 1990) in cultured mammalian cells.

Unscheduled DNA synthesis (UDS) reflects the genotoxicity of carcinogens and is one measure of the rate of DNA repair. Consequently, detection of UDS is utilized as a technique of mutagenicity testing. Several investigators have tried to detect induction of UDS by PUVA *in vitro*, *in vivo* and *in vivo - in vitro*. Baden *et al.* (1972) and Kaye *et al.* (1980) reported induction of UDS by PUVA in human fibroblasts *in vitro* by liquid scintillation counting and autoradiography, and by liquid scintillation counting, respectively. On the other hand, induction of UDS by PUVA has never been detected *in vivo* or *in vivo - in vitro*. Epstein and Fukuyama (1975) failed to demonstrate induction of UDS by PUVA in hairless mouse epidermis *in vivo* by autoradiography. Hönigsmann *et al.* (1981) failed to detect induction of UDS by PUVA in healthy human epidermis *in vivo - in vitro* by autoradiography. Bioulac *et al.* (1980), and Bishop and Abel (1985) also found no induction of UDS by PUVA in skin from psoriatic patients *in vivo - in vitro* by autoradiography. We recently reported induction of UDS by skin carcinogens (Mori *et al.*, 1999) and UV irradiation (Mori *et al.*, 2000a), and we also evaluated age-related changes of DNA repair capacity in the epidermis of hairless mice by assaying induction of UDS after UV-B irradiation (Mori *et al.*, 2000b), using a modification of the *in vivo - in vitro* liquid scintillation counting methods which had been developed for UDS studies in the glandular stomach, forestomach, colon and liver of rats (Furihata *et al.*, 1984, 1985; Furihata and Matsushima, 1987; Sawada *et al.*, 1989; Ohsawa *et al.*, 1993; Furihata and Matsushima, 1995). The feature of this method, as modified for the epidermis, is the evaluation of UDS induction in terms of the UDS index. The use of the UDS index is effective for the detection of UDS induction under conditions where total DNA synthesis (TDS) is reduced owing to inhibition of cell proliferation. The UDS index is calculated after simultaneous measurements of DNA synthesis in the presence and absence of hydroxyurea (HU), which is an inhibitor of replicative DNA synthesis (RDS) (Adams and Lindsay, 1967). In the present study, we examined whether our

in vivo - in vitro liquid scintillation counting method is able to detect induction of UDS by PUVA in hairless mouse epidermis. A UV-absorbing glass filter was used in order to eliminate wavelengths below 320 nm. We first tried to find the optimal induction time of UDS after PUVA in a time-course study, and then examined the optimal UV-A dose in a dose-response study. Under the conditions thus identified, we investigated the optimal dose of 8-MOP. The induction of UDS by PUVA *in vivo - in vitro* was then evaluated under the optimal conditions.

MATERIALS AND METHODS

Chemicals

8-MOP (CAS No. 298-81-7) was obtained from Aldrich Chemical Co. (Milwaukee, WI). [$\text{CH}_3\text{-}^3\text{H}$] Thymidine ($[^3\text{H}]\text{dThd}$, 78 Ci/mmol (2.90 TBq/mmol)) was from ICN Radiochemicals (Irvine, CA).

Ultraviolet radiation sources

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 4.90 mW/cm². These fluorescent tubes were fitted on a medical ultraviolet irradiation apparatus (Dermaray M-DMR-100, Eisai Co., Tokyo), and ultraviolet intensities were measured using a UV-radiometer (UVR-305/365-D, Eisai 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. To ensure sufficient penetration of 8-MOP into the epidermis, tape-stripping treatments were given five times with five 24 mm $\times$ 100 mm pieces of cellulose tape (Nichiban Co., Cat. No. LP-24, Tokyo, Japan) within 30 sec on the dorsal region. 8-MOP dissolved in acetone was applied once onto two areas (upper and lower back) by using a ring with a diameter of 15 mm (Nakanishi Company, Inc., Cat. No. Hatome 30, Tokyo, Japan), then mice were placed under the medical ultraviolet irradiation apparatus, and irradiated with UV-A. Animal experiments were conducted in accordance with the guidelines for animal experimentation 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, 2000a). Mice underwent euthanasia at the indicated times after irradiation. Two skin pieces of approximately 177 mm² from the skin to which 8-MOP and subsequent UV-A had been applied 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/ml streptomycin, 100 units/ml penicillin G and 370 kBq [³H]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/ml streptomycin and 100 units/ml penicillin G, and then incubated at 37 °C for 30 min in 3 ml of phosphate-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 tetrasodium 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, 2000a). 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 (Furihata *et al.*, 1985). Aliquots of 50 µl of the supernatant were incubated with 100 µl of 2 M 3,5-diaminobenzoic acid dihydrochloride (Tokyo Kasei Kogyo Co., Tokyo, Japan) at 37 °C for 30 min and 600 µl of 0.6 N perchloric 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 ($\times 100$) of the amount of DNA synthesis in the presence of HU to that in its absence.

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

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

Statistical analysis

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

RESULTS

Time-course of UDS induction by PUVA

DNA synthesis in the presence of HU was not significantly changed at 1, 2, 4 and 24 hr after 1×10^5 J/m² UV-A irradiation with 0.001% 8-MOP application compared to the control acetone application (Fig. 1A), and DNA synthesis in the absence of HU was significantly decreased at 1 and 2 hr (Fig. 1B). The UDS index was significantly increased at 1, 2 and 24 hr, showing a maximal 2.9-fold increase at 24 hr (Fig. 1C), while it showed no change in the case of control acetone application. Subsequent studies were assayed at 24 hr after irradiation.

UV-A dose-related response of UDS induction by PUVA

DNA synthesis in the presence of HU was unchanged after 0.5×10^5 , 1×10^5 , 2×10^5 and 4×10^5 J/m² UV-A irradiation at 24 hr with 0.001% 8-MOP application compared to the control acetone application (Fig. 2A), and DNA synthesis in the absence of HU

was significantly decreased after 1×10^5 and 4×10^5 J/m² UV-A irradiation (Fig. 2B). The UDS index was significantly increased after 1×10^5 J/m² UV-A irradiation, showing a 2.2-fold increase (Fig. 2C), while it showed no change in the case of control acetone application. For the subsequent 8-MOP dose-related study, the dose of 1×10^5 J/m² of UV-A irradiation was chosen.

Fig. 1. Time-course of UDS induction by PUVA. The skins were obtained at the indicated times after 1×10^5 J/m² UV-A irradiation with acetone or 0.001% 8-MOP application. Fig. A, B and C show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean $\pm$ S.E. for groups of three mice. Asterisks indicate a significant difference from the control group at each time; * $p < 0.05$, ** $p < 0.01$.

8-MOP dose-related response of UDS induction by PUVA

DNA synthesis in the presence of HU was unchanged at 0.0005, 0.001 and 0.002% 8-MOP application at 24 hr after 1×10^5 J/m² UV-A irradiation compared to the control acetone application (Fig. 3A), and DNA synthesis in the absence of HU was significantly decreased at 0.001 and 0.002% 8-MOP application (Fig. 3B). Thus, the UDS index was increased over this dose range, showing a 2.4-fold increase at 0.002% (Fig.

Fig. 2. UV-A dose-related response of UDS induction by PUVA. The skins were obtained at 24 hr after UV-A irradiation with acetone or 0.001% 8-MOP application. Fig. A, B and C show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean $\pm$ S.E. for groups of three mice. Asterisks indicate a significant difference from the control group at each UV-A dose; * $p < 0.05$, ** $p < 0.01$.

3C). Neither 0.001% 8-MOP application in the absence of UV-A nor UV-A irradiation without 8-MOP caused any change (compared to acetone application in the absence of UV-A) in the DNA synthesis in the presence or absence of HU, or in the UDS index.

Fig. 3. 8-MOP dose-related response of UDS induction by PUVA. The skins were obtained at 24 hr after $1 \times 10^5 \text{ J/m}^2$ UV-A non-irradiation or irradiation with acetone or 8-MOP. Fig. A, B and C show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean $\pm$ S.E. for groups of five mice. 'Ns's and asterisks indicate no significant difference and a significant difference, respectively, between the indicated groups; $^{\text{Ns}}p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

DISCUSSION

We were able to detect induction of UDS by PUVA in the epidermis *in vivo* - *in vitro* by using our method. However, the increase of the UDS index was less than 3-fold. Thus, the induction of UDS by PUVA was far smaller than that by 4-nitroquinoline 1-oxide (4NQO), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or UV-B irradiation (Mori *et al.*, 1999, 2000a). In studies *in vivo* or *in vivo* - *in vitro* by an autoradiographic method, previous investigators have failed to detect induction of UDS (Epstein and Fukuyama, 1975; Bioulac *et al.*, 1980; Hönigsmann *et al.*, 1981; Bishop and Abel, 1985). Our success in the detection of UDS induction *in vivo* - *in vitro* in the present study might have been due to the carefully designed experimental procedures that we employed. For example, we used a UV-absorbing glass filter in order to eliminate the wavelength range of UV-B. In other studies, a UV-absorbing glass filter was not used (Bioulac *et al.*, 1980), or a plate glass with a low UV-absorbing effect was used (Bishop and Abel, 1985). The presence of UV-B would increase the control level of DNA synthesis, and thereby mask slight induction of UDS by PUVA. The specific activity of radiolabeled dThd is also an important factor. We used radiolabeled dThd with a high specific activity of 78 Ci/mmol, whereas in the four studies that failed to detect induction of UDS, the specific activity of the radiolabeled dThd was in the range from 5 to 25 Ci/mmol. The use of radiolabeled dThd with low specific activity would lead to difficulties in sensitive detection of UDS induction. In addition, we first performed a time-course study, and then a dose-response study for UV-A to find optimal conditions for induction of UDS, and confirmed an 8-MOP dose-related response in a subsequent dose-response study. None of the other groups employed such methodology. The use of appropriate experimental conditions is critical for the detection of low levels of UDS induction.

The evaluation of UDS induction in terms of the UDS index was one of the key points in the detection of UDS induction by PUVA. In general, it is difficult to detect a small amount of UDS in the skin with liquid scintillation counting, because induction of UDS in the skin is accompanied by a decrease of TDS (Mori *et al.*, 1999, 2000a, 2000b). PUVA caused a clear decrease of TDS. The calculation of the UDS index allows the detection of UDS induction under such conditions. We previously reported the relationship between the change of DNA synthesis and induction of UDS in

dose-response studies (Mori *et al.*, 1999). We concluded that i) parallel changes of DNA synthesis in the presence and absence of HU while maintaining a UDS index of approximately two is indicative of non-induction of UDS; ii) cases in which DNA synthesis in the presence of HU was increased and that in its absence was decreased were regarded as clear induction of UDS, and in this case, the UDS index is greatly increased; iii) when DNA synthesis in the presence of HU was unchanged and that in its absence was decreased, this was also regarded as indicating induction of UDS; in such a case, the UDS index shows a small increase. The changes of DNA synthesis in the presence and absence of HU in PUVA came under the last category, indicating slight induction of UDS by PUVA.

We administered 8-MOP by topical application subsequent to tape-stripping, before UV-A irradiation. We did not try other administration routes, such as p.o. and i.p., because the carcinogenicity of 8-MOP orally administered in PUVA seems to be far lower than that after topical application (Griffin, 1959; Langner *et al.*, 1977), and i.p. administration has not been used for humans.

In the time-course study (Fig. 1), the control acetone application increased DNA synthesis in the presence and absence of HU at 24 hr after UV-A irradiation. This reflected the cell proliferation after the tape-stripping treatments. However, since the control acetone application did not produce an increase of the UDS index at 24 hr, we were able to assay induction of UDS by PUVA at this time point. Induction of UDS by PUVA was observed throughout the period, except at 4 hr. This implies that DNA excision repair takes place gradually over a long period. Reduction of UDS at 4 hr might be explained in terms of excision repair capacity of epidermal cells for two types of photoadducts between 8-MOP and DNA, *i.e.*, monoadducts and cross-links. Either the 3,4-double bond in the pyrone ring or the 4',5'-double bond in the furan ring of 8-MOP photoreacts with the 5',6'-double bond of the pyrimidine in DNA in the presence of UV-A, producing 3,4-cyclobutane monoadducts or 4',5'-cyclobutane monoadducts, respectively. Further photoreaction of these monoadducts leads to further ring formation with another nearby pyrimidine on the opposite DNA strand, and results in the formation of diadducts, *i.e.*, cross-links (Musajo *et al.*, 1967a, 1967b; Dall'Acqua *et al.*, 1970). Stern (1989) noted that repair response to 8-MOP in human fibroblasts primarily involves the removal of monoadducts, and cross-links appear to per-

sist. Chandra *et al.* (1974) investigated the removal of adducts in guinea pig skin using radiolabeled psoralen, which is an analogue of 8-MOP, and confirmed that monoadducts are more easily removed from DNA than cross-links. Thus, since the removal rates of monoadducts and cross-links differ from each other, it seems natural that the pattern of UDS induction should be biphasic. Reduction of UDS at 4 hr might correspond to completion of excision of monoadducts, and reinduction of UDS at 24 hr might reflect removal of cross-links.

In the UV-A dose-response study (Fig. 2), induction of UDS was not observed at the doses of 2×10^5 and 4×10^5 J/m², though the dose of 1×10^5 J/m² induced UDS. Prolonged UV-A irradiation is said to convert monoadducts into cross-links (Dall'Acqua *et al.*, 1970), and, according to Seki *et al.* (1978), cross-links are responsible for lethal effects rather than mutation induction. The lack of induction of UDS at the doses of 2×10^5 and 4×10^5 J/m² might reflect further cross-link formation and a decrease of excision repair capacity owing to an increase of lethal action.

In the 8-MOP dose-response study (Fig. 3), induction of UDS by PUVA was small. The pattern of DNA synthesis in the presence and absence of HU, and the change of UDS index by PUVA were similar to those in response to diepoxybutane (DEB), 7,12-dimethylbenz[a]anthracene (DMBA) and benzo[a]pyrene (B[a]P) in a previous study (Mori *et al.*, 1999). Kleinau *et al.* (1997) reported that UV-B accelerates the 8-MOP adduct repair process, and the repair of thymine dimers and 8-MOP adducts together is probably easier and faster to perform than the repair of monoadducts and cross-links generated by PUVA. Their result suggests that the use of a solar simulator, which emits not only UV-A but also UV-B, might increase UDS more than the use of UV-A. Further studies of the effects of various lamps and other photomutagens on the induction of UDS should contribute to the establishment of the photo-UDS assay as a test method to detect photomutagenic or photocarcinogenic chemicals.

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