INDUCTION OF UNSCHEDULED DNA SYNTHESIS IN HAIRLESS MOUSE EPIDERMIS BY SKIN CARCINOGENS

Masaaki MORI, Hiroshi KOBAYASHI, Chiyomi SUGIYAMA, Yoshio KATSUMURA and Chie FURIHATA

1Life Science Research Center, Shiseido Co., Ltd., 1050 Nippari-cho, Kohoku-ku, Yokohama-shi, Kanagawa 223-8552, Japan
2Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

(Received April 1, 1999; Accepted June 8, 1999)

ABSTRACT — Induction of unscheduled DNA synthesis (UDS) in hairless mouse epidermis by six chemicals was determined in an in vivo - in vitro assay by using a liquid scintillation counting method. Test chemicals were applied once onto two areas of the back of female hairless mice after stripping of the stratum corneum with adhesive tape to enhance skin penetration. After exposure, the skin samples were taken and cultured in a medium containing [3H]thymidine with or without hydroxyurea (HU, an inhibitor of replicative DNA synthesis). DNA of the epidermis was extracted, and incorporation of [3H]thymidine into DNA and the DNA content was determined with a liquid scintillation counter and a fluorescence spectrophotometer, respectively. Induction of UDS by chemicals was judged by calculation of the UDS index [(the ratio of DNA synthesis in the presence of HU to that in its absence) × 100]. A good correlation between UDS induction and organ specificity of carcinogens was observed. 4-Nitroquinoline 1-oxide, a skin carcinogen used as a positive control, induced a dose-dependent increase in the UDS index of approximately 12-fold at 2 hr after exposure, while 1,2-epoxydecane, a non-skin carcinogen applied as a negative control, did not increase the UDS index. Four other skin carcinogens induced dose-dependent increases in the UDS index; N-methyl-N'-nitro-N-nitrosoguanidine and diepoxybutane at 2 hr after exposure, and 7,12-dimethylbenz[a]anthracene and benzo[a]pyrene at 24 hr after exposure. The results suggest that UDS is a good marker of the genotoxicity of skin carcinogens.

KEY WORDS: Unscheduled DNA synthesis, Hairless mouse epidermis, Skin carcinogen

INTRODUCTION

The skin is highly exposed to environmental chemicals, mutagens and carcinogens, but the presence of the stratum corneum makes it difficult to detect damage to DNA. Only a few genotoxicity test methods are available for the skin. Assaying unscheduled DNA synthesis (UDS) is one such method. UDS, which reflects DNA damage, generally occurs shortly after exposure to the inducer. To assist in maximizing detection of UDS, precise application of the test chemical onto a fixed skin area is required. Ishikawa et al. (1982) succeeded in detecting UDS in mouse skin with an autoradiographic method. In their protocol, areas of mouse skin were first clamped off with ring-shaped tongue forceps, and were injected s.c. with an isotonic aqueous solution containing a carcinogen and [3H]thymidine; the tissue was later excised and processed for autoradiography. They demonstrated induction of UDS by 4-nitroquinoline 1-oxide (4NQO), 4-hydroxyaminoquinoline 1-oxide, methyl methanesulfonate and 1-methyl-1-nitrosourea, which are known skin carcinogens (Shirasu, 1963; Parish and Searle, 1966; Graff et al., 1967; Druckrey et al., 1970).

In order to avoid such complicated techniques, animal discomfort and the administration of a radioac-
tive compound, we tried to develop an easier method. Methods using liquid scintillation counting for in vivo - in vitro assay of UDS in the glandular stomach, forestomach, colon and liver of rats have been described previously (Furihata et al., 1984, 1985; Furihata and Matsushima, 1987; Furihata et al., 1988; Oshima et al., 1989; Sawada et al., 1989; Ohsawa et al., 1993; Asakura et al., 1994; Furihata and Matsushima, 1995). These methods examined DNA synthesis simultaneously in the presence and absence of hydroxyurea (HU), which is an inhibitor of replicative DNA synthesis (RDS) (Adams and Lindsay, 1967). In a recent study (Ohsawa et al., 1993), UDS induction was expressed by calculating the equation [UDS] = ([DS(±HU)] ± k[DS(−HU)])b(1−k) in the glandular stomach of rats: [UDS] is UDS at time t, [DS(±HU)] is the observed DNA synthesis with HU at time t, k is a constant for inhibition of RDS by HU, and [DS(−HU)] is the observed DNA synthesis without HU at time t. The value of k is determined by the observed results at time 0: k = [DS(±HU)]/[DS(−HU)]. Glandular stomach carcinogens induced UDS without a remarkable reduction of RDS. However, skin carcinogens induced marked inhibition of RDS in hairless mouse epidermis. Therefore we could not apply the equation for the glandular stomach to the epidermis. Instead, we adopted the UDS index, defined as the ratio (× 100) of DNA synthesis in the presence of HU to that in its absence. Using this parameter, we examined the induction of UDS in the epidermis of hairless mice treated with (i) the skin carcinogen 4NQO, (ii) 1,2-epoxydodecane (EDD), which is not a skin carcinogen, and (iii) four other skin carcinogens, viz., N-methyl-N'-nitro-N-nitrosoguanidine (MNGN), diepoxybutane (DEB), 7,12-dimethylybenz[a]anthracene (DMBA) and benzo[a]pyrene (B[a]P). The results suggest that UDS is a good marker of the genotoxicity of skin carcinogens.

MATERIALS AND METHODS

Chemicals

MNGN (CAS No. 70-25-7) and EDD (CAS No. 2855-19-8) were obtained from Aldrich Chemical Co. (Milwaukee, WI), DEB (CAS No. 1464-53-5), 4NQO (CAS No. 56-57-5) and DMBA (CAS No. 57-97-6) were from Tokyo Kasei Kogyo Co. (Tokyo, Japan), B[a]P (CAS No. 50-32-8) was from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). [CH3-3H]Thymidine (1H)dThd, 2.37 - 3.11 TBq/mmol) was from ICN Radiochemicals (Irvine, CA).

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 allow chemicals to penetrate directly into the epidermis, tape-stripping treatments were given five times with five 24 mm×100 mm pieces of cellulose tape (Nichiban Co., Cat. No. LP-24, Tokyo, Japan) within 30 seconds on the dorsal region. Test chemical dissolved in acetone was applied once onto two areas (upper and lower back) by using a circle ring with a diameter of 15 mm (Nakanishi Company, Inc., Cat. No. Hatome 30, Tokyo, Japan). In this way, the solutions were distributed uniformly over the designated areas. All applications were performed under ether anesthesia. 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 Shiseido Research Center for Animal Welfare.

Preparation of epidermal extracts

Mice underwent euthanasia at the indicated times after treatment. The two portions of the skin where a test chemical 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 of streptomycin/ml, 100 units of penicillin G/ml 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 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 phosphate-buffered saline, and the epidermis was separated with tweezers. DNA was extracted from the epidermis by a modification (Furihata et al., 1984) of the method of Schmidt and Thannhauser (1945), and Schneider (1957). 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 10% trichloroacetic acid, homogenized in 1 ml of 1 N potassium hydroxide, and incubated at 37°C overnight to hydrolyze RNA. On the next

Vol. 24 No. 3

NII-Electronic Library Service
day, the supernatant was neutralized with hydrochloric acid, and DNA and protein were precipitated with 5% trichloroacetic acid. The precipitate was homogenized in 700 μl of 5% trichloroacetic acid and incubated at 80°C for 30 min to hydrolyze DNA. DNA fraction was obtained as the supernatant after centrifugation.

**Measurements of DNA synthesis**

Aliquots of 500 μl of the supernatant with HU and 100 μl of that without HU were dissolved in ACS II scintillant (Amersham Corp., Arlington Heights, IL) and incorporation of [3H]dTdh 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-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 shows the ratio (×100) of the amount of DNA synthesis in the presence of HU to that in its absence.

UDS index = [DS(±HU)] / [DS(−HU)] × 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 ± standard error (S.E.). Statistical significance was determined by the use of Student’s t (Welch) test (Gad and Weil, 1982).

**RESULTS**

**Optimal conditions for assay of DNA synthesis**

In a preliminary study, five sequential episodes of tape-stripping were concluded to be optimal on the basis of histological observation of the skin (data not shown). Analysis of DNA synthesis in the presence of HU showed that 5 to 15 mM HU in the culture medium inhibited RDS by up to 98% in the epidermis of untreated control mice (Fig. 1); a concentration of 10 mM HU was used in subsequent studies. We also examined the effects of skin area, culture time and [3H]dTdh concentration on incorporation of [3H]dTdh into DNA (data not shown). Decrease of the skin area increased the incorporation of [3H]dTdh into DNA. However, incorporation of [3H]dTdh into DNA was constant from 177 to 346 mm² skin area with and without HU, and the UDS index did not change appreciably with skin area. For subsequent studies, skin pieces of approximately 177 mm² were used. Incorporation of [3H]dTdh into DNA linearly increased for up to 2 hr in the culture and this time point was selected for further assays. Incorporation of [3H]dTdh into DNA also depended on [3H]dTdh concentration. DNA synthesis in the presence and absence of HU increased in proportion to [3H]dTdh concentration, while the UDS index values were constant over the range of 370 to 1480 kBq [3H]dTdh/ml. A [3H]dTdh concentration of 370 kBq/ml was chosen for subsequent studies.

**Influence of tape-stripping treatments and acetone on DNA synthesis**

It was necessary to determine whether tape-stripping had any effect in our UDS assay. Fig. 2A shows that DNA synthesis in the presence and absence of HU was not affected during 4 hr after 5 episodes of tape-
stripping, but was increased at 24 hr. However, the UDS index was unchanged (Fig. 2B). Since test chemicals were dissolved in acetone, we also studied the effect of acetone on UDS (data not shown). Acetone application after tape-stripping treatments did not alter DNA synthesis.

**Induction of UDS by 4NQO**

DNA synthesis in the presence of HU was increased at 1 and 2 hr after 0.05% 4NQO application compared to the control acetone application, and DNA synthesis in the absence of HU was decreased during 1 to 24 hr. The UDS index was increased from 1 to 3 hr (data not shown). The stimulation of UDS and the inhibition of RDS 2 hr after application were shown to be dose-dependent over the range of 0.05-0.2% 4NQO (Fig. 3A). Thus, the UDS index values increased over this dose range, showing an approximately 12-fold increase at 0.2% (Fig. 3B).

**Non-induction of UDS by EDD**

EDD is a non-skin carcinogen and a direct-acting chemical. Since the direct-acting skin carcinogen MNNG induced UDS 2 hr after application as described below (Fig. 5), we examined the dose-dependency of EDD action on DNA synthesis in hairless mouse epidermis 2 hr after application. Fig 4A shows that EDD inhibited DNA synthesis both in the presence and absence of HU. The inhibition was dose-dependent for 0.5, 1 and 2% EDD, with a 50% decrease at 2% EDD. However, the UDS index was unchanged over this dose range (Fig. 4B).

**Induction of UDS by MNNG**

DNA synthesis in the presence of HU was increased at 1, 2 and 3 hr after 0.2% MNNG application compared to the control acetone application, and DNA synthesis in its absence was decreased during 1 to 24 hr. The UDS index was increased at 1 to 3 hr (data not shown). The DNA synthesis and the UDS index 2 hr

![Fig. 2. Influence of tape-stripping treatments on DNA synthesis and the UDS index in hairless mouse epidermis. The skins were obtained at the indicated times after five episodes of tape-stripping treatment. Fig. A and B show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean±S.E. of a group of five mice. Asterisks indicate a significant difference from the control group; **p<0.01, ***p<0.001.](image1)

![Fig. 3. Dose-dependent induction of UDS by 4NQO. The skins were obtained at 2 hr after application of 4NQO. Fig. A and B show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean±S.E. of a group of five mice. Asterisks indicate a significant difference from the control group; *p<0.05, **p<0.01, ***p<0.001.](image2)
after MNNG application were dose-dependent for 0.1, 0.2 and 0.4% MNNG (Fig. 5A, 5B). The UDS index was increased over this dose range, showing an approximately 8-fold increase at 0.2% (Fig. 5B).

**Induction of UDS by DEB**
DNA synthesis in the presence of HU was only slightly increased at 2 and 4 hr after 1.0% DEB application compared to the control acetone application, and DNA synthesis in its absence was decreased during 1 to 24 hr. The UDS index was increased during 1 to 24 hr (data not shown). DNA synthesis in the presence of HU was unchanged after 0.2, 0.4 and 0.8% DEB, while DNA synthesis in its absence showed a dose-dependent decrease at 2 hr after application (Fig. 6A). Thus, the UDS index was increased over this dose range, showing a more than 2-fold increase at 0.8% (Fig. 6B).

**Induction of UDS by DMBA**
DNA synthesis in the presence of HU was increased at 24 hr after 0.5% DMBA application compared to the control acetone application, and DNA synthesis in its absence was decreased at 16, 24 and 48 hr. The UDS index was increased at 24 hr (data not shown). DNA synthesis in the presence of HU was unchanged by 0.25, 0.5 and 1% DMBA, while DNA synthesis in the absence of HU showed a dose-dependent decrease at 24 hr after application (Fig. 7A). Thus, the UDS index was increased over this dose range, showing an approximately 4-fold increase with 1% DMBA (Fig. 7B).

**Fig. 4.** Effect of EDD on DNA synthesis. The skins were obtained at 2 hr after application of EDD. Fig. A and B show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean ± S.E. of a group of five mice. Asterisks indicate a significant difference from the control group; *p<0.05, **p<0.01.

**Fig. 5.** Dose-dependent induction of UDS by MNNG. The skins were obtained at 2 hr after application of MNNG. Fig. A and B show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean ± S.E. of a group of five mice. Asterisks indicate a significant difference from the control group; *p<0.05, **p<0.01, ***p<0.001.
HU was unchanged by 0.25, 0.5 and 1% B[a]P, while DNA synthesis in its absence was decreased to approximately 40% at 24 hr after application (Fig. 8A). The UDS index changed over this dose range, showing a more than 2-fold increase with 0.5% B[a]P (Fig. 8B).

**DISCUSSION**

The present study shows that our method based on liquid scintillation counting is useful to detect UDS caused by chemicals in the skin. Our procedure incorporated tape-stripping to remove the stratum corneum in order to enhance percutaneous absorption of the applied chemical. The use of a circle ring made it possible to distribute chemicals uniformly over the test sites. To validate the method, we examined 4NQO, which had previously been shown to induce UDS in mouse skin by means of an autoradiographic method (Ishikawa et al., 1982), and we confirmed its activity by our method.

The determination of incorporation of [3H]dThd

**Fig. 6.** Dose-dependent induction of UDS by DEB. The skins were obtained at 2 hr after application of DEB. Fig. A and B show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean±S.E. of a group of five mice. Asterisks indicate a significant difference from the control group; *p<0.05, **p<0.01, ***p<0.001.

**Fig. 7.** Dose-dependent induction of UDS by DMBA. The skins were obtained at 24 hr after application of DMBA. Fig. A and B show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean±S.E. of a group of five mice. Asterisks indicate a significant difference from the control group; *p<0.05, **p<0.01, ***p<0.001.

**Fig. 8.** Dose-dependent induction of UDS by B[a]P. The skins were obtained at 24 hr after application of B[a]P. Fig. A and B show DNA synthesis in the presence and absence of HU, and the UDS index. Values are the mean±S.E. of a group of five mice. Asterisks indicate a significant difference from the control group; *p<0.05, **p<0.01, ***p<0.001.
into DNA by using liquid scintillation counting is faster and easier than by using autoradiography. In some previous studies using liquid scintillation counting, only DNA synthesis in the presence of HU was assayed to determine UDS (Martin et al., 1978; Tsutsui et al., 1984). However, HU does not completely inhibit RDS (Furuta et al., 1984; Sawada et al., 1989). In our study, HU inhibited RDS up to approximately 98% in hairless mouse epidermis. In order to obtain a more accurate measure of the UDS-inducing activity of test chemicals, we adopted simultaneous determination of DNA synthesis in the presence and absence of HU, and we evaluated UDS induction in terms of the calculated UDS index, [ratio of DNA synthesis in the presence of HU to that in its absence] × 100).

In preliminary studies to optimize the assay, we observed that DNA synthesis in the presence of HU amounted to approximately 2% of DNA synthesis in the absence of HU, and showed a similar pattern to DNA synthesis in the absence of HU. We considered that parallel change of DNA synthesis in the presence and absence of HU while maintaining a UDS index of approximately two should be indicative of non-induction of UDS. 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. 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.

4NQO and MNNG both increased DNA synthesis in the presence of HU, and decreased DNA synthesis in its absence, resulting in a marked increase in the UDS index values. On the other hand, DEB, DMBA and B[a]P did not increase DNA synthesis in the presence of HU, but reduced DNA synthesis considerably in the absence of HU, so that overall the UDS index values increased marginally. EDD, used as a negative control, decreased DNA synthesis dose-dependently in the presence and absence of HU, and the UDS index did not increase. This may reflect the fact that EDD is not a skin carcinogen (Van Duuren et al., 1967), though it causes malignant lymphoma (Kotin and Falk, 1963).

Of the 5 carcinogens evaluated for UDS induction in hairless mouse epidermis, MNNG and DEB are direct-acting skin carcinogens (Van Duuren et al., 1963; Sugimura et al., 1966a; Takayama et al., 1971). These chemicals induced UDS in the epidermis at 2 hr after exposure. On the other hand, 4NQO, DMBA and B[a]P are indirect-acting carcinogens (Klein, 1956; Wynder et al., 1957; Parish and Searle, 1966; Kato et al., 1970; DiGiovanni et al., 1983; Shimada et al., 1989). 4NQO is activated by D,T-diaphorase, which plays an important role in reductive metabolic activation of procarcinogens (Kato et al., 1970). This activation is rapid and UDS was detected at 2 hr after exposure in our study. DMBA and B[a]P are activated by the IA subfamily of cytochrome P-450-dependent mono-oxygenases (Shimada et al., 1989). These chemicals induced UDS in the epidermis at 24 hr after exposure. Thus, differences in modes and rates of carcinogen metabolism can explain the observed differences in induction times and magnitudes of UDS. In mammals, the liver possesses higher D,T-diaphorase and cytochrome P-450 1A1 activities than any other organ (Sugimura et al., 1965, 1966b; Guengerich and Mason, 1979; Mukhtar and Brickers, 1981). Sugimura et al. (1966b) reported that D,T-diaphorase activity in skin homogenate of the mature Donryu albino rat was approximately one-tenth of that in liver homogenate. On the other hand, Mukhtar and Brickers (1981) reported that cytochrome P-450 1A1 activity in the skin of Wistar neonatal rats was approximately one-seventieth of that in the liver. DiGiovanni et al. (1986) reported that excision repair of DMBA active metabolites combined with DNA in mouse skin is carried out slowly over a long time, and this is also the case for repair of B[a]P-DNA adducts in mouse epidermis (Nakayama et al., 1984). Slow and low level UDS induction by DMBA and B[a]P might be related to the slow reaction velocity of cytochrome P-450 1A1 and slow excision repair over an extended period.

Cytochrome P-450 1A1 in mouse skin is localized in the superficial layer of the dermis, or in the epidermis (Wiebel et al., 1975; Akin and Norred, 1976; Thompson and Slaga, 1976; Mukhtar and Brickers, 1981; Brickers et al., 1982). Therefore, we believe that the tape-stripping procedure that we employed to remove the stratum corneum would not have influenced the specific activity of cytochrome P-450 dependent mono-oxygenase.

Since cytochrome P-450 1A1 in the skin is an inducible enzyme, as it is in the liver (Wiebel et al., 1975; Thompson and Slaga, 1976; Mukhtar and Brickers, 1981), one might expect that skin pretreatment with a cytochrome P-450 1A1 inducer would significantly enhance the induction of UDS. However, it was reported that pretreatment with a cytochrome P-450 1A1 inducer decreased DNA adduct formation and the incidence of tumors (Lutz, 1979). The reason for this is not clear.

Reduction of RDS by chemicals signifies toxicity to cells. However, it is often seen that chemicals are
assayed at toxic doses in mutagenicity tests. For example, the mouse lymphoma cell gene mutation assay is performed in the range of 10 to 20% relative cell survival, i.e., at toxic doses (Clive et al., 1995; Sofuni et al., 1996). Induction of UDS at doses of test chemicals that reduce RDS seems to be a characteristic of the skin, so data on toxicity (i.e., reduction of RDS) should be helpful for selecting optimal doses for UDS assay.

In summary, six carcinogens were tested for induction of UDS in hairless mouse epidermis using a liquid scintillation counting method. Five skin carcinogens induced UDS in the epidermis and a non-skin carcinogen did not. The results suggest that UDS is a good marker of genotoxicity of skin carcinogens.

ACKNOWLEDGMENTS

The authors are grateful to Dr. J. Fitzgerald and Dr. W. R. S. Steele for their critical reading of the manuscript.

REFERENCES


Unscheduled DNA synthesis in mouse epidermis by skin carcinogens.


M. MORI et al.


