Induction and Persistence of Pyrimidine Dimers in the Epidermal DNA of Two Strains of Hairless Mice

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SUMMARY

The ultraviolet-light induction of DNA damage has been measured in the epidermis of hairless mice with the use of damage-specific endonucleases from Micrococcus luteus. The rates of induction of endonuclease-sensitive sites in HRS/J/Anl and SKH:hairless-1 mice were $6.1 \pm 0.5 \times 10^{-11}$ and $6.5 \pm 0.8 \times 10^{-11}$ dalton/J/sq m from a FS40 fluorescent sun lamp (280 to 400 nm), respectively. Enzymatic photoreactivation with yeast photoreactivating enzyme showed that ~80% of the endonuclease-sensitive sites were cyclobutyl pyrimidine dimers. In both strains of mice the pyrimidine dimers remained in high-molecular-weight DNA for 24 hr after irradiation. These data show that mouse epidermal cells in vivo have little or no capacity for the excision repair of pyrimidine dimers.

INTRODUCTION

The UV-B (280- to 320-nm) portion of the UV spectrum has been shown to be mutagenic (26) and carcinogenic (9). However, the precise action spectrum, molecular lesions, and the role of repair processes in photocarcinogenesis are not known. We have initiated tumor induction studies using different spectra of UV, alone or in combination with chemical sensitization, in an attempt to determine the carcinogenic effect of different spectra of UV and to determine the contribution of DNA damage in oncogenesis. In studies of the UV induction of skin carcinomas in mice photosensitized by 8-methoxypsoralen, we have observed a marked strain difference in susceptibility even though the induction of psoralen-mediated, DNA interstrand cross-links and cutaneous damage was similar (10, 15). A similar strain difference in susceptibility also has been observed for the time of appearance of tumors induced by UV alone (P. D. Forbes, personal communication). One possible explanation for strain-dependent differences in susceptibility is that there is also a strain-dependent difference in the capacity of epidermal cells to repair DNA damage. Thus, we wished to determine whether there existed a strain difference in the capacity of epidermal cells to excise UV-induced damage from their DNA.

The removal of UV-induced pyrimidine dimers from DNA has been reported from human fibroblasts grown in culture (18), and, recently, Setlow et al. (22) and Lehmann (13) reported low levels (10 to 15%) of dimer excision after relatively low exposures of UV (5 to 10 J/sq m at 254 nm) in mouse fibroblasts in vitro. Peleg et al. (17) reported that mouse embryo fibroblasts in early passages exhibited repair, but cells from later passages did not. Furthermore, Bowden et al. (2) have reported that mouse epithelial cells in vivo excised 60% of the dimers induced by relatively high exposures at 254 nm.

The ability of cells to excise UV-induced pyrimidine dimers from their DNA was first reported in bacterial cells (3, 20). The model proposed for this excision-repair process involves (11, 19): (a) incision near the pyrimidine dimer by a damage-specific endonuclease; (b) removal of the dimer and adjacent nucleotides by an exonuclease; (c) polymerization of undamaged nucleotide into the single-strand gap; and (d) ligation to join the replaced sequence with the contiguous DNA strand.

We have used damage-specific endonucleases from Micrococcus luteus to assay for the induction and fate of UV-induced ESS in the DNA of mouse epithelial cells in vivo for 2 strains of hairless albino mice. Furthermore, we have demonstrated that ~80% of the lesions recognized by these endonucleases are pyrimidine dimers.

MATERIALS AND METHODS

Animals

The 2 strains of hairless albino mice, HRS/J/Anl and SKH:hr-1, used for these studies have been described previously (15). Female mice, 8 to 14 weeks of age, were used in these experiments.

Radionuclide Labeling of Epidermal DNA

Injections i.p. Groups of mice received 4 to 6 i.p. injections of 100 μCi of [methyl-3H]thymidine ([3H]TdR, 10 to 20 Ci/mole; New England Nuclear, Boston, Mass.) at 6-hr intervals.

Air Pouch Injections. A 5-ml air pouch was introduced underneath the skin of the back and injected with [3H]TdR as described by Bowden and Boutwell (1) and Bowden et al. (2).

The abbreviations used are: ESS, endonuclease-sensitive sites; [3H]TdR, tritiated thymidine; PR, photoreactivation.
Short-Term Culturing of Excised Mouse Epidermis. Strips of skin, approximately 2 x 3 cm, were excised from the middorsum region, and the upper 0.4-mm surface was removed from the dermal layer with a Norelco keratotome (Storz Surgical Instruments, St. Louis, Mo.). The epidermal layer was subsequently floated, dermal side down, onto RPMI-1640 medium (Microbiological Associates, Bethesda, Md.) supplemented with 15% fetal calf serum (Microbiological Associates) and 100 μg of gentamicin per ml (Schering Corp., Kenilworth, N.J.). [3H]TdR (specific activity, 10 to 13 Ci/mmole) at a concentration of 2 μCi/ml was introduced into the medium, and the skin was then incubated at 37° in a humidified 5% CO₂ atmosphere for 18 to 22 hr.

Light Sources and Exposure Conditions
Subsequent to the labeling of epidermal DNA, the dorsal epidermis of mice was exposed to varying fluences of UV from 1 of 2 different light sources. In the in vivo experiments, mice were exposed to UV within individual lucite exposure chambers (6 x 4 x 3 cm) overlaid by a 1-sq cm mesh wire screen. Excised dorsal epidermis that had been labeled in vitro was exposed to UV while floating, dermal side downward, on the surface of ice-cold 0.1 M Tris buffer, pH 8.0, that contained 0.01 M EDTA and 0.2 M NaCl. Radiation at 254 nm was obtained from a germicidal lamp at a fluence rate of 4 watts/sq m, as determined with a calibrated vacuum thermopile (FT 17.1, Hilger and Watts; Des Plaines, Ill.) in combination with a microvoltmeter (Model 150B; Keithley Instruments, Cleveland, Ohio). The 2nd light source used was a Westinghouse FS40 fluorescent sun lamp that emits wavelengths between 280 and 400 nm with a peak at 313 nm (10). The fluence rates from the FS40 sun lamp were 2.0 watts/sq m for whole-animal irradiations and 3.3 watts/sq m for in vitro irradiations, as determined with a calibrated YSI-Kettering Model 65 radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Isolation of Epidermal DNA
Labeled DNA from epithelial cells was isolated as previously described (15) and dialyzed against 0.05 M Tris buffer, pH 7.0.

Assay for ESS
Damage-specific endonucleases were prepared from M. luteus according to the method of Carrier and Setlow (5) with modifications as proposed by W. L. Carrier (personal communication); spray-dried cells (0.6 g; Miles Laboratories, Kankakee, Ill.) were suspended in 30 ml of 0.2 M sucrose and 0.01 M Tris buffer (pH 8.0) and incubated for 45 min at 30° in the presence of 200 μg of lysozyme per ml (Sigma Chemical Co., St. Louis, Mo.). Thirty ml of cold distilled water were added and the lysate was vigorously mixed for 30 min at ice-bath temperature. Four ml of 10% streptomycin sulfate were added over a 30-min interval, with constant stirring to precipitate nucleic acids. Cell debris and precipitated nucleic acids were removed by centrifugation at 12,000 x g for 30 min. Ammonium sulfate (20 g) was added to the resulting supernatant (50 ml) at ice-bath temperature over a 30-min interval, with continuous stirring. The mixture was stirred for an additional hr at 0°, and the resulting precipitate containing the endonuclease activity was collected by centrifugation (12,000 x g for 30 min). The precipitate was dissolved in 10 ml of 0.01 M Tris buffer, pH 8.0, and stored as 0.5-ml aliquots at −20°.

Endonuclease-sensitive site assays were performed by mixing 0.2 ml (−2 μg) of mouse epidermal DNA in phosphate buffer with 20 μl of "assay mix" (see below) and 20 μl of M. luteus extract and incubating at 37° for 15 min. The reaction was stopped by the addition of 50 μl of 1 M NaOH, and the incubation was continued for an additional 30 min to assure complete denaturation of the DNA. Additional samples were treated in the same manner without the M. luteus extract. The assay mix used in these experiments was to inhibit nonspecific nucleases and contained 0.05 M EDTA, 100 μg of crude yeast tRNA per ml (Miles Laboratories), and 200 μg of sonically disrupted calf thymus DNA per ml in 0.05 M phosphate buffer, pH 7.0).

Sedimentation of DNA in Alkaline Sucrose Gradients
The entire sample of alkaline denatured DNA was layered onto a 4.6-m1 alkaline 5 to 20% sucrose gradient that contained 0.7 M NaCl, 0.3 M NaOH, and 0.01 M EDTA. The gradients were centrifuged in a Beckman SW 50.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). Centrifugation speeds and times are presented in the appropriate chart legends. The distributions of radioactivity in the gradients and molecular weights were determined as previously described (15). Number-average molecular weights were calculated from 0.5 x the weight-average molecular weights, since direct calculation of number-average molecular weights is very sensitive to small fluctuations in the low-molecular-weight region of the gradients.

Pyrimidine Dimer Assays
Mouse epidermal DNA from excised skin, labeled and irradiated in vitro, was subjected to 2 cycles of the DNA extraction procedure described previously (15). A repeat of the extraction procedure eliminated the streaking we had observed on paper chromatograms of hydrolysates of DNA that had been subjected to a single extraction sequence. Purified [3H]DNA (～200,000 cpm) in 1 ml of phosphate buffer was precipitated by the addition of trichloroacetic acid to a final concentration of 10%. The precipitate was collected by centrifugation and washed twice with 95% ethanol and once with diethyl ether. Hydrolysis of the DNA and subsequent 2-dimensional paper chromatography were as described by Carrier and Setlow (4).

Photoreactivation Experiments with Yeast Photoreactivating Enzyme
Two ml of DNA in 0.05 M potassium phosphate buffer, pH 7.0, were mixed with 0.1 ml of assay mix (see above) and 0.3 ml of a yeast photoreactivating enzyme preparation that was generously provided by Dr. John S. Cook. The enzyme
preparation was obtained following phosphocellulose column chromatography (8). One-half of the mixture was placed in a spot dish at 37° and exposed for 1 hr to 365-nm light from a Magnaflux mercury vapor lamp with a fluence rate of 50 watts/sq m. The remaining half was held in the dark for 1 hr at 37°. After the treatments the DNA was reisolated and assayed for pyrimidine dimers and ESS.

RESULTS

Induction of ESS in Epidermal DNA with the 254-nm and FS40 Light Sources. We have used damage-specific endonucleases in an extract of M. luteus as a convenient and sensitive assay for the in vivo induction of base damage in mouse epidermal DNA by UV radiation. Chart 1 presents sedimentation profiles from alkaline sucrose gradients of DNA extracted from epithelial cells of the SKH:hr-1 strain of mice that were labeled by air pouch injections of [methyl-3H]thymidine, prior to exposure to 254-nm radiation or the FS40 sun lamp (280 to 400 nm). The sedimentation profiles and single-strand molecular weights of the extracted DNA without further treatment were comparable (~10 x 10^6 daltons), regardless of the source of UV (Chart 1A). However, if prior to sedimentation in alkaline, the DNA from irradiated animals was incubated with an extract from M. luteus, the DNA did not sediment as far into the gradient (Chart 1B). In the absence of UV exposure, incubation with the M. luteus extract resulted in little or no shift in the sedimentation profiles (data not shown). It is apparent from the profiles presented in Chart 1B that, following treatment with the M. luteus extract, the distribution of radioactivity does not approximate a gaussian distribution and is skewed toward the high-molecular-weight region of the gradient. DNA extracted from 254-nm light, suggesting a non-random distribution of damage. Thus, epidermal DNA from animals exposed to either source of radiation contains DNA alterations sensitive to the damage-specific endonucleases.

Note: The text contains a formula and a chart for the rates of induction and persistence of ESS in epidermal DNA of the HRS/J/Anl and SKH:hr-1 Strains of Mice. The chart illustrates the number of ESS/10^6 daltons induced by different fluences of UV radiation from the FS40 sun lamp and the rates of induction and persistence of ESS in epidermal DNA of the SKH:hr-1 strain with and without the presence of the M. luteus extract.

The text also mentions that the number of ESS/10^6 daltons is determined by linear regression analysis. We observed that the FS40 sun lamp induced 6.5 ± 0.8 x 10^-11 and 6.1 ± 0.5 ESS/10^6 daltons.

Similar treatment of DNA from mice killed 24 hr after irradiation yielded ~3.8 ESS/10^6 daltons (Chart 2B). Thus, it would appear that 10% of the sites had been removed during the 24 hr after irradiation. However, the errors associated with these determinations would probably make it difficult to measure a loss of ESS that did not exceed 25% of the initial number induced. For a better estimate of DNA damage in both strains of mice, killed immediately or 24 hr after irradiation, fluence-response curves were plotted in Chart 3 as numbers of ESS/10^6 daltons. Slopes for the curves were determined by linear regression analysis. We observed that the FS40 sun lamp induced 6.5 ± 0.8 x 10^-11 and 6.1 ± 0.5 ESS/10^6 daltons.

Pyrimidine Dimers in Epidermal DNA

Since skewed distributions of radioactivity increase the difficulty in calculating an average molecular weight for the population of molecules, and since we presently are using the FS40 sun lamp in tumor induction studies, the remainder of the experiments reported herein on UV-induced DNA damage in mouse epithelial cells were carried out with the FS40 sun lamp.

Rates of Induction and Persistence of ESS in Epidermal DNA of the HRS/J/Anl and SKH:hr-1 Strains of Mice. Mice labeled in their DNA by i.p. injections were exposed to graded fluences from the FS40 sun lamp and killed immediately or 24 hr later. Labeled DNA was extracted from the epidermis and sedimented in alkaline sucrose gradients prior to or after treatment with the M. luteus extract. Typical sedimentation profiles are presented in Chart 2. Number-average molecular weights calculated from these profiles were used to calculate the number of ESS induced in epidermal DNA of the SKH:hr-1 strain by 4 x 10^2 J/sq m of UV. For epidermal DNA extracted immediately after irradiation, we calculate that treatment with the damage-specific endonucleases has reduced the single-stranded molecular weight of the extracted DNA from 17 x 10^6 to 9.9 x 10^6 (Chart 2A). Thus, 4 x 10^2 J/sq m of UV has induced

\[ \frac{10^6}{9.9 \times 10^6} \cdot \frac{1}{17 \times 10^6} \]

4.2 ESS/10^6 daltons.

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x 10^{-11} ESS/dalton/J/sq m in epidermal DNA of the SKH:hr-1 (Chart 3A) and HRS/J/Anl (Chart 3B) mice, respectively, when determined immediately after irradiation. Slopes of the curves obtained for animals killed 24 hr after irradiation were calculated to be $5.2 \pm 1.3 \times 10^{-11}$ and $6.5 \pm 0.8 \times 10^{-11}$ ESS/dalton/J/sq m for the SKH:hr-1 and HRS/J/Anl mice, respectively. Thus, it would appear that there is no apparent difference in the rate of induction of ESS in the 2 strains of mice. In addition, there was no apparent loss of these sites during 24 hr in either strain.

**Evidence Obtained with Short-Term Culturing of Excised Epidermis That ESS Induced with the FS40 Sun Lamp Are Pyrimidine Dimers.** The use of damage-specific endonucleases to detect DNA damage provides a convenient and sensitive technique, but the broad spectrum of damage recognized by the extracts (5, 6, 16, 25) used in these studies does not allow us to specify the particular type of damage we have assayed in epidermal DNA from mice exposed to the FS40 sun lamp. Although the emission spectrum from this light source encompasses wavelengths that are known to produce cyclobutyl pyrimidine dimers in DNA (21, 24), we could at best only speculate that this source would produce pyrimidine dimers in epidermal DNA at a rate equivalent to the observed rate of induction of ESS. Thus, a series of experiments was performed to measure, chromatographically, the induction of pyrimidine dimers in epidermal DNA. For these experiments, the upper 0.4 mm of skin was excised from mice and placed in cell culture medium containing [methyl-3H]thymidine at 37°C for 18 to 22 hr (see "Materials and Methods"). This short-term culturing of epidermis enhanced the radionuclide labeling of epidermal DNA to provide sufficient radioactivity to measure pyrimidine dimers directly by paper chromatography. Following prelabeling, the layer of skin was removed from the medium, floated on the surface of ice-cold buffer, and exposed to graded fluences of UV from the FS40 source. Labeled DNA was isolated from epidermal cells and hydrolyzed, and the hydrolysate was chromatographed in 2 dimensions according to the method of Carrier and Setlow (4). The radioactivity in the dimer region of the chromatogram was determined and expressed as a percentage of the total radioactivity applied to the chromatogram. These percentages are plotted in Chart 4 as a function of fluence. From the slope of the dimer induction curve, we calculate that 1 $\times 10^4$ J/sq m resulted in 0.2% of the labeled thymine occurring in pyrimidine dimers. Lehmann (12) has previously calculated for mouse fibroblasts that 0.019% radioactivity as dimers would equal $\sim$1 dimer/10^7 daltons of DNA. Therefore, 0.2% radioactivity as dimers would equal 10.5 dimers/10^7 daltons in DNA. Aliquots of DNA samples assayed for pyrimidine dimers also were assayed for ESS. From the slope of the ESS induction curve (Chart 5), we calculate $0.9 \times 10^{-10}$ sites induced/dalton/J/sq m, an induction rate equivalent to that observed for pyrimidine dimers in the epidermal DNA. Equivalent rates of induction, unfortunately, do not rule out other types of damage, induced at the same rate as dimers, as being the ESS sites we have measured.

To determine whether the ESS we observed were in fact pyrimidine dimers, a sample of DNA that contained 0.33% of the labeled thymine as dimers was treated with yeast PR enzyme and exposure to 365-nm light. Enzymatic PR results in the monomerization of the dimer in situ (19). The PR treatment resulted in a 67% reduction in the number of pyrimidine dimers (Chart 4). In addition, following comparable PR treatments, we observed a 53% reduction in the number of ESS (Chart 5). We conclude, therefore, that at least 80% of the ESS induced in epidermal DNA with the FS40 source are pyrimidine dimers. Furthermore, because the induction of ESS in epidermal DNA from skin that had been labeled and irradiated in vivo (Chart 3) is only 30% less...
tumor induction with this treatment (10). Molecular studies we observed a marked strain difference in susceptibility to the SKH:hr-1 and HRS/J/Anl strains of mice with UV in whole-animal irradiations is predominantly UV-induced pyrimidine dimers in DNA for at least 24 hr after induction.

DISCUSSION

In our previous studies on the induction of skin tumors in the SKH:hr-1 and HRS/J/Anl strains of mice with UV in combination with the photosensitizer 8-methoxypsoralen, we observed a marked strain difference in susceptibility to tumor induction with this treatment (10). Molecular studies on the induction of cross-linkages in epidermal DNA (1) produced results that were comparable for both strains of mice (15). Thus, different levels of DNA damage resulting from comparable treatments could not be proposed as an explanation for the different tumor induction responses. However, the possibility remained that, although comparable levels of damage are initially induced, a strain difference existed in the capacity of epidermal cells to repair damage to their DNA. A reduced capacity for repair could possibly result in an enhanced sensitivity to the tumor-inducing treatments. Credence was given this possibility in view of DNA repair studies that have been conducted on human skin fibroblasts derived from patients who suffer a genetic disorder called xeroderma pigmentosum. One symptom of this condition is an abnormally high occurrence of skin tumors, which are presumed to be UV induced. Fibroblasts from patients with xeroderma pigmentosum grown in culture exhibit reduced capacity for the repair of UV-induced DNA damage, for example, excision repair (7), postreplication repair (14), and PR (23). Although a causal relationship has not been shown to exist between reduced repair capac-

Pyrimidine Dimers in Epidermal DNA

The results we have reported here show that (a) the FS40 sun lamp (280 to 400 nm) induced damage in mouse epidermal DNA that, following extraction, is recognized by the damage-specific endonucleases from M. luteus (Chart 1). In addition, the rates of induction are comparable for both strains of mice, i.e., ~6.3 × 10^{-11} daltons/J/sq m (Chart 3); (b) 80% of the ESS are UV-induced cyclobutyl pyrimidine dimers as shown by PR with yeast PR enzyme and long-wavelength UV; and (c) both strains of mice exhibit, as has been reported for mouse fibroblasts in vitro, little or no capacity for the excision of pyrimidine dimers from epidermal DNA. Although the latter finding is in contradiction with the observations by Bowden et al. (2), our results suggest a possible explanation for the discrepancy. The induction of pyrimidine dimers in mouse epithelial cells with 254-nm light results in a nonrandom distribution of damage in the epidermal DNA. This is apparent from the skewed sedimentation profiles obtained from alkaline sucrose gradients after treatment with the damage-specific endonucleases (Chart 1B) and suggested from the observation by Bowden et al. (2) that the yield of dimers saturates at a level 10-fold smaller than expected, indicating that 90% of the cells were not irradiated and 10% were heavily irradiated. If the heavily damaged molecules are specifically degraded to acid-soluble products during a 24-hr "repair" period, analysis of the remaining, less damaged acid-insoluble DNA would reveal lower numbers of dimers per unit mass of DNA than one would obtain for DNA analyzed immediately after irradiation. Therefore, what would appear to be a selective removal of dimers by excision repair from an entire population of DNA molecules is in fact the selective degradation of a heavily damaged subpopulation of molecules. Presumably, this would be less likely to occur in the experiments we have reported on here since (a) the exposures we used for the repair studies induced considerably fewer dimers per unit mass of DNA; 1 to 6/10^8 daltons in our study (Chart 3) as compared with 35 to 250/10^8 daltons in the study of Bowden et al. (2); and (b) the dimers induced with the FS40 sun lamp appear to be more randomly distributed in the epidermal DNA.

* The epidermal DNA obtained from mice in this experiment (Chart 1) was labeled by the air-pouch injection procedure used by Bowden et al. (2) in their studies on the induction of pyrimidine dimers in epidermal DNA.
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Our studies indicate that neither different rates of induction of pyrimidine dimers in epidermal DNA nor different capacities for the excision repair of dimers can be proposed as a basis for the strain difference in susceptibility to UV-induced tumorigenesis. However, it cannot be ruled out that a strain difference exists for other repair processes, e.g., postreplication repair, or that a UV-induced, nondimer lesion is subject to excision repair in one strain and not the other.

Finally, our studies with short-term culturing of excised epidermis indicate that this method can be used to facilitate radionuclide labeling of epidermal DNA and yet maintain the UV transmission properties of the epidermis. The short-term culture may allow us to investigate other repair parameters of epithelial cells such as postreplication repair and repair replication.

REFERENCES

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