Immunohistochemical Detection of Carcinogen-DNA Adducts and DNA Repair in Mouse Skin

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4-Hydroxyaminoquinoline 1-oxide (4HAQO) and (±)-trans-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BP-DE)-DNA adducts were immunohistochemically demonstrated in the nuclei of mouse skin using antibodies directed against carcinogen (4HAQO or BP) modified DNA. The specificity of the immunostaining was confirmed by several tests, including preincubation of the antibody with carcinogen modified DNA or related molecules, and digestion of the sections with DNase.

Subcutaneous injection of 4HAQO dissolved in isotonic solution into an isolated portion of the mouse skin clamped off with ring-shaped forceps resulted in dose-dependent generation of DNA adducts in the nuclei of epithelial cells, fibroblasts, and panniculus carnosus cells. BP-DNA adducts could also be similarly detected dose-dependently in the nuclei of skin cells after local application of BP-DE. Nuclear staining was absent in animals injected with isotonic solution alone, and the intensity of staining correlated well with the level of unscheduled DNA synthesis (UDS) demonstrated autoradiographically after treatment with 4HAQO. Killing of mice at different time points after a single injection of 4HAQO revealed a gradual decrease in the intensity of the staining. Thus the postulated generation and repair of DNA adducts can be followed at the cellular level using the presently described method. *J Invest Dermatol 92:275S–279S, 1989*

The initial step in chemical carcinogenesis is probably the covalent binding of carcinogen to DNA of the target cell. Several carcinogen-DNA adducts have been isolated and their structures identified [1–4]. Although studies on the fate of carcinogen adducts in the nucleic acids of exposed cells have usually focused on biochemical and radioimmune assays, more recently specific antibodies have been raised against DNA adducts with several kinds of carcinogens. Immunologic determination of such adducts has been performed by a number of laboratories [5–15]. Thus development of immunohistochemical techniques has allowed visualization of carcinogen-DNA adduct formation in individual cells or in strata of whole tissues [16–22]. With this method it should be possible to gain a better understanding of the relevance of DNA binding and DNA repair to the mechanisms underlying chemical carcinogenesis; for example, in the skin.

Antibodies to DNA adducts of two skin carcinogens are the subject of the present report. 4HAQO, a proximate form of 4-nitroquinoline 1-oxide [23], which was first demonstrated to induce tumor development in the skin by Nakahara et al [24], reacts with DNA to yield four kinds of adducts: one 4HAQO-adenine and 3 4HAQO-guanine structures [25]. The second carcinogen, benzo[a]pyrene (BP), which is a worldwide environmental contaminant and may be directly involved in human carcinogenesis, has the anti-form of BP-DE, the ultimate binding species [26,27].

Specific antibodies raised against 4HAQO-DNA adducts [28,29] or BP-DNA adducts [30] were used for immunohistochemical detection of nuclear carcinogen-DNA adducts in paraffin embedded mouse skin sections after application of carcinogens in vivo. Application of the method was extended to include studies on adduct removal from skin after local or systemic exposure to 4HAQO.

MATERIALS AND METHODS

Anti-Carcinogen-DNA Adduct Antibody Antibody specific for 4HAQO or BP modified DNA was raised and characterized in the same way as previously described [28,30]. Briefly, 4HAQO-DNA or BP-DNA was electrostatically bound to methylated bovine serum albumin (mBSA), and rabbits were immunized with an emulsion of carcinogen-DNA-mBSA complex with complete or incomplete Freund's adjuvant. Blood samples were taken after the final injection of the antigen mixture without adjuvant. Serum was refined and collected as the IgG fraction. Antibody specificity was verified by an enzyme-linked immunosorbent assay. The antibodies recognized carcinogen (4HAQO or BP) modified DNA but not unmodified DNA, other carcinogen modified DNA or free carcinogen derivatives.

Immunohistochemical Detection of Carcinogen-DNA Adducts in the Skin Female ICR mice (8 weeks old), weighing 26 to 30 g, were obtained from Charles River Japan (Atsugi-shi, Japan). For local treatment of mouse skin with carcinogen, the technique used was essentially the same as that reported earlier [31,32] and thus is only briefly described here. Animals were anesthetized with sodium pentobarbital, and the skin of their backs was shaved with electric clippers. Tongue forceps (ring shaped, 20 mm in internal

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Abbreviations:
ABC: avidin-biotin peroxidase complex
BP: benzo[a]pyrene
BP-DE: (±)-trans-7,8-dihydroxy-9,10-epoxy-7,8,9,10 tetrahydrobenzo[a]pyrene
DAB: 3,3′-diaminobenzidine, tetrahydrochloride
4HAQO: 4-hydroxyaminoquinoline 1-oxide
4NQO: 4-nitroquinoline 1-oxide
PBS: phosphate buffered saline
UDS: unscheduled DNA synthesis

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diameter), of the type normally used clinically, were used to clamp off a double fold of skin, with as little stretching as possible. This clamped-off area of skin was then marked with a pen. Immediately after the skin was clamped off, 0.5 ml of solution containing different concentrations of carcinogen was injected s.c. into the clamped-off region with a fine needle. To confirm the specificity of the immunohistochemical staining, 1-methyl-1-nitrosourea (5×10^{-3} M), methylmethane sulfonate (15×10^{-2} M), or Ringer solution alone was injected. The mice with shaved skin were also exposed to UV irradiation at a dose of 2600 J/m² from a sunlamp with three 20-watt fluorescent tubes (Toshiba FL 20 S.E. sunlamp) with emission at 270 to 440 nm and a peak at 312 nm.

Three mice each received a single i.v. injection of 4HAQO (Iwai Kagaku Co., Tokyo, Japan) solution (dissolved in a small amount of 0.025 N HCl solution) at doses of 0.5, 5, or 20 mg/kg body weight to determine the distribution of adduct throughout the body. The mice were killed 3 h later by decapitation, and the skin and the other various organs were quickly removed.

To ascertain any correlation between dose dependencies of staining and UDS, four animals each were then treated with different concentrations of 4HAQO (0.75, 2.5, 7.5, 25, 75, or 250×10^{-6} M), and [methyl-^{3}H] thymidine [50μCi/0.5 ml/mouse; specific activity 75 Ci/mmol, 100 μCi/ml (New England Nuclear, Boston, MA)]. For studies on the time course of DNA repair, groups of four mice each received an injection of 4HAQO (75×10^{-6} M) and were killed 2, 24, 48, or 72 h after treatment. Each experiment included at least four animals treated with Ringer solution alone.

Using the same method as for 4HAQO, three doses of BP-DE (5×10^{-4}, 1×10^{-4}, 2×10^{-5} M: ITT Research Institute, Chicago, IL, under the auspices of Dr. Longfellow, National Cancer Institute, Bethesda, MD) and Ringer solution alone were injected s.c. into the clamped-off region.

Immediately after injection, the mice were kept at 35°C for 60 min and the forceps were removed. The animals were killed 2 h later except those in the repair-time course study.

Tissue specimens were fixed in 10% neutral formaldehyde solution for 18 h at room temperature. To obtain identical staining conditions, control and carcinogen-exposed tissues were embedded in the same paraffin block in all experiments. Paraffin-embedded material was sectioned at 3 to 4 μm, and the sections were mounted on glass slides coated with poly(L-lysine). After incubation in methanol with 0.3% H_2O_2 for 30 min, dehydration through a graded ethanol series was performed.

To avoid any cross-reaction with RNA, the sections were first treated with RNase A (200 μg/ml from bovine pancreas; Sigma Chemical Co., St. Louis, MO: pretreated at 70°C for 10 min to inactivate DNase). The sections were then treated with 2 N HCl at room temperature for 5 h and incubated in 3% normal goat serum containing PBS. Binding of anti-carcinogen-DNA adduct antibody was carried out at 4°C for 16 h and visualized by the avidin-biotin peroxidase complex method (Vectastain kit from Vector Laboratories, Inc., Burlingame, CA) with the use of 0.01% H_2O_2 and 0.05% DAB (incubation in 0.05 M Tris buffer pH 7.2, for 10 min). After washing in deionized water, the sections were dehydrated through graded ethanol and xylene, and mounted.

To confirm the specificity of immunohistochemical staining, antibody was substituted either with normal rabbit sera or specific antibody absorbed with an excess of carcinogen modified DNA or unmodified DNA. Other sections were incubated with DNaseI (from bovine pancreas; Sigma Chemical Co.) diluted to 500 μg/ml with PBS added to 3 mM $MgCl_2$ at 37°C for 4 h before staining with the antibody.

Autoradiographic procedures were performed as previously described [31,32]. Skin sections stained for 4HQAO-DNA adduct by specific antibody were dip-covered with NR-M_2 autoradiographic emulsion (Konishiroku Photo Co., Tokyo, Japan) and exposed at 4°C for 4 weeks. Because the range of variability in the grain numbers was small throughout any particular sample, grains were counted consecutively (in one direction) on more than 200 basal cells starting from a randomly selected point. The background grains on control sections were counted in the same way.

RESULTS

Immunohistochemical Procedure and Specificity Tests In order to determine the most suitable condition for detection of carcinogen-DNA adducts, skin samples from mice that received a single s.c. injection of 0.5 ml 4HAQO solution (2.5×10^{-4} M) into the clamped-off region were used. Cryostat sections and paraffin sections from materials fixed in acetone, methanol, Carnoy's solution, paraformaldehyde, or 10% neutral formaldehyde were tested for binding of anti-4HAQO-DNA adduct antibody. Most fixatives proved unsuitable, because nuclei of control sections demonstrated nonspecific staining. Moreover, some sections deteriorated during incubation in 2 N HCl. Paraffin sections fixed in 10% neutral formaldehyde, however, had none of these disadvantages and provided the best morphologic preservation (Figs 1 and 2).

A few features of the staining process have to be stressed. For example, treatment of tissue sections with HCl was necessary for detection of adducts. Optimal HCl concentration, temperature, and incubation time were investigated, and it was found that a treatment period of 5 hours with 2N HCl at 25°C was associated with a maximum amount of DAB precipitate. Treatments for longer than 5 hours with 2N HCl decreased staining intensity. Treatments of sections with RNase before exposure to the specific antibody eliminated cytoplasmic staining but did not affect specific nuclear staining.

Under the chosen experimental conditions, anti-4HAQO-DNA adducts antibody did not bind in detectable amounts to tissues of unexposed mice (Figs 1*B* and 2*B*). Control rabbit sera also did not react with tissues of 4HAQO-exposed mice (data not shown). In sections from mouse skin exposed to sunlamp UV (2600 J/m²),

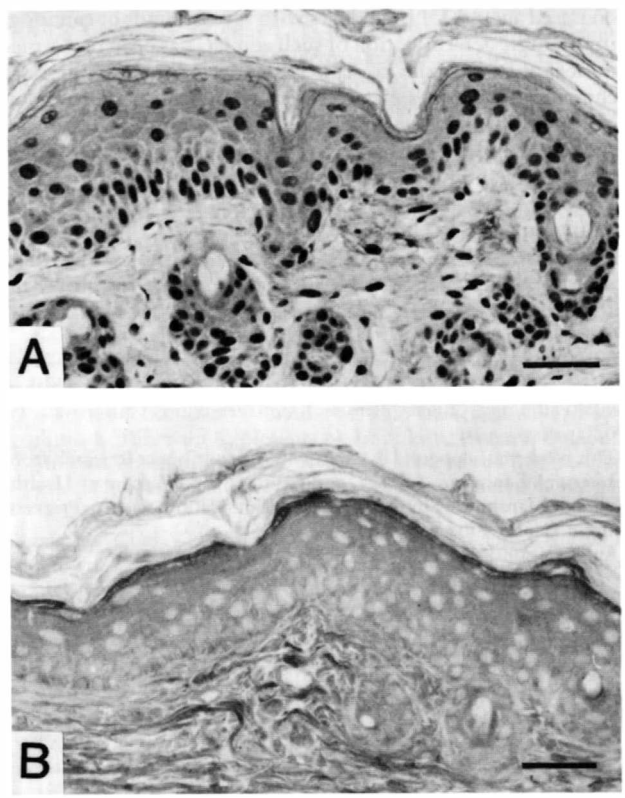

Figure 1. Nuclear immunohistochemical staining of 4HAQO-DNA adducts in skin sections from mice exposed to 4HAQO *(A)* but not Ringer solution *(B)*. 4HAQO solution [75×10^{-6} M] *(A)* or Ringer solution *(B)* was injected s.c. into regions of skin region clamped with tongue forceps. The sections were stained with anti-4HAQO-DNA adduct antibody using the ABC method. (*Bar:* 50 μm).

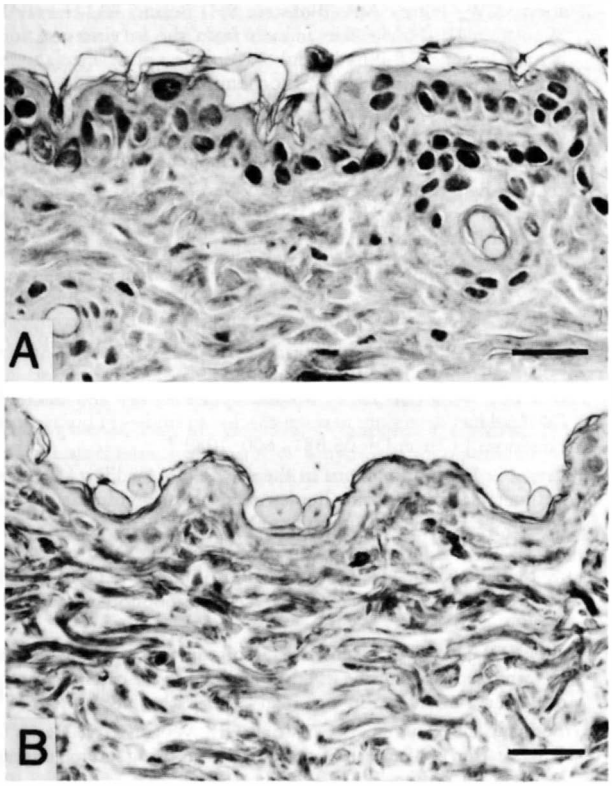

Figure 2. Nuclear immunohistochemical staining of BP-DNA adducts in skin sections from mice exposed to BP-DE *(A)* but not Ringer solution *(B)*. BP-DE solution [2×10^{-5} M] *(A)* or Ringer solution *(B)* was injected s.c. into regions of skin region clamped with tongue forceps. The sections were stained with anti-BP-DNA adduct antibody using the ABC method. (*Bar:* 25 μm).

1-methyl-1-nitrosourea (5×10^{-3} M), or methyl methane sulfonate (1.5×10^{-2} M), nuclear staining was negative with the anti-4HAQO-DNA adducts antibody (data not shown). Nuclear staining was eliminated when the antibody was mixed with an excess of 4HAQO modified DNA prior to incubating sections with the antibody, but was hardly changed when the antibody was mixed with an excess of unmodified-DNA (data not shown). When sections were preincubated with DNase before addition of anti-4HAQO-DNA adduct antibody, nuclear staining was eliminated (data not shown). These data indicate that recognition by the antibody is highly specific for the type of DNA damage caused by 4HAQO in vivo.

Essentially the same results were gained with anti-BP-DNA adduct antibody.

4HAQO-DNA Adducts in Mouse Skin When 4HAQO solution was injected s.c. into an isolated portion of the mouse skin clamped off with ring-shaped forceps, immunohistochemical staining of DNA adducts was observed in nuclei of epithelial cells, fibroblasts, and panniculus carnosus cells (Fig 1*A*). The level of staining observed 3 h after injection of 0.75, 2.5, 7.5, or 250×10^{-6} M 4HAQO was dose dependent with no significant nuclear binding found for the lowest dose (data not shown). UDS was clearly seen as silver grains over the nuclei of epithelial cells. A clear correlation between 4HAQO dose and levels of UDS and nuclear staining (amount of 4HAQO-DNA adducts) was demonstrated.

Mice killed at 2, 24, 48, or 72 h after injection of 4HAQO revealed a gradual decrease in the intensity of staining of epithelial cells between 2 and 72 h (Fig 3), suggesting that repair of the adducts had taken place.

Mice that received a single i.v. injection of 4HAQO solution at 5 or 20 mg/kg and were killed 3 h thereafter demonstrated a predominance of nuclear staining in organs that were considered targets for 4HAQO-carcinogenesis: skin, pancreas, uterus, vagina, colon, lung, and trachea (data not shown).

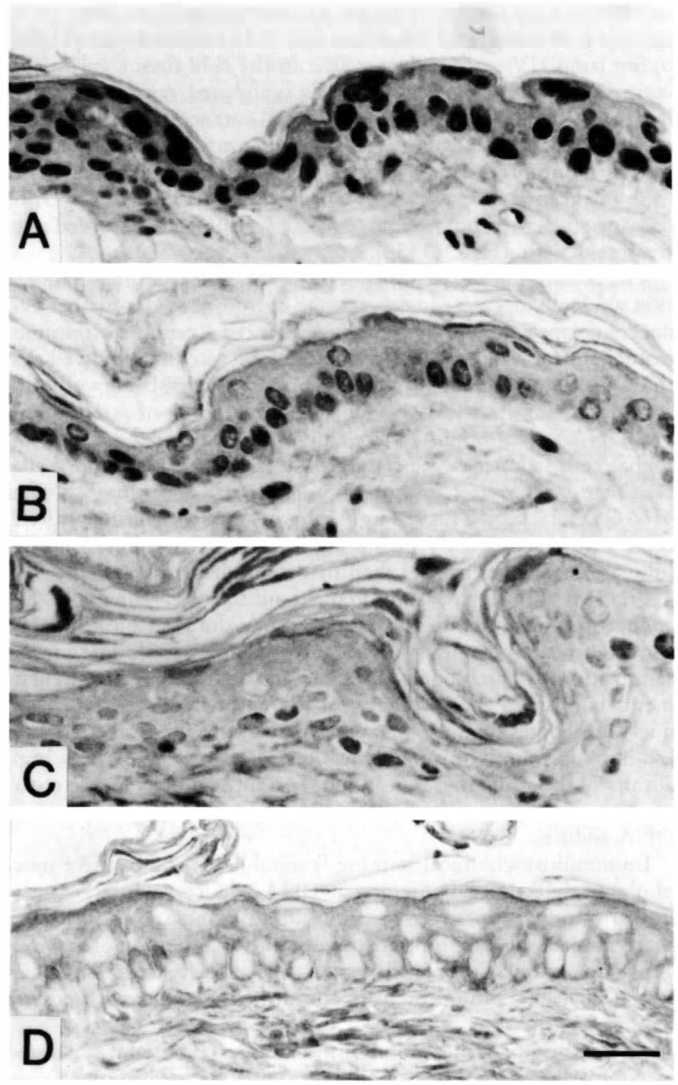

Figure 3. DNA repair rate of 4HAQO-DNA adducts in mouse skin. 4HAQO solution [75×10^{-6} M] was injected s.c. into regions of skin region clamped with tongue forceps and the skin was removed 2 h *(A)*, 24 h *(B)*, 48 h *(C)*, or 72 h *(D)* thereafter. Note the decreasing intensity of staining with time (*Bar:* 24 μm) 37.

BP-DNA Adducts in Mouse Skin Mice that received a s.c. injection of BP-DE solution into an isolated portion of the skin clamped off with ring-shaped forceps and who were killed 3 h thereafter demonstrated a predominance of nuclear staining in skin cells (Fig 2). Dose-dependent staining of nuclei of the skin was also observed at 3 h after injection of 50, 10, and 2×10^{-5} M DP-DE (data not shown).

DISCUSSION

DNA repair systems are regarded as among the most important in organisms. However, relatively little is known about DNA repair in vivo in the skin, because few appropriate methods for studying it are available. UV-induced DNA repair has been studied by direct measurement of pyrimidine dimers in the skin [33,34], but while this method is specific, it cannot give information on the location of DNA repair within individual skin cells.

Another approach has been our autoradiographic measurement of DNA repair at the single cell level in skin in vivo after treatment with UV [32] or chemical carcinogen [31]. We used this technique

and clarified the following points. a) Epithelial cells are more proficient (3 to 4 times) than fibroblast in DNA excision repair [31,32]; b) Sunlamp UV reaches deeper sites in the skin than a germicidal lamp [32]; c) DNA repair synthesis is induced rapidly after UV irradiation and continues for 48 h [32]; d) cutaneous hair can screen about 90% of the UV energy [32] and melanin is less important than hair for photoprotection against UV irradiation in guinea pig skin [35]; and e) aged animals cannot repair extensive UV-induced DNA damage efficiently, but no age-associated changes are observed after treatment with 4HAQO [36].

The present finding that the intensity of adduct staining in mouse skin gradually decreased with passage of time provides further evidence of repair of carcinogen-DNA adducts. Combined immunohistochemical staining and autoradiographic methods showed that the UDS levels and DNA adducts formation correlated well with comparable sensitivities. Although the initial levels of anti-carcinogen-DNA adduct binding were similar in both fibroblast and epithelial cells, adducts were removed far more quickly in epithelial cells than in fibroblasts where staining was still apparent 72 h after 4HAQO injection. This strongly suggests rapid repair in epithelial cells, which agrees with the finding that UDS levels in the epithelium are 3–5 times greater than that found in fibroblasts.

Previous studies have indicated the utility of carcinogen-DNA adduct specific antibodies as tools to probe the relevance of DNA adducts to carcinogenesis *in vivo* and *in vitro* [5–28]. The precise localization of carcinogen-DNA adducts in the series of experiments confirmed the specificity of the anti-carcinogen-DNA adduct antibodies raised. Enzyme-linked immunosorbent assay [28,30] and immunohistochemical tests [29] revealed a very high affinity of the antibodies for carcinogen-DNA adducts and no cross-reaction with unmodified DNA or other carcinogen modified DNA adducts.

Immunohistochemical staining is suitable for comparative morphologic detection of carcinogen-DNA adducts, while enzyme-linked immunosorbent assays and radioimmunoassays [5–15] are more sensitive and appropriate for absolute quantitation of carcinogen-DNA adducts. Nevertheless, a clear positive correlation between nuclear staining intensity and dose of carcinogen used was observed in the skin.

The sensitivity of the immunohistochemical method was assessed by comparing the results gained with those obtained from radioactive analysis using [^{3}H]-4HAQO. The lowest limit of detection for the staining was 3–5 adducts per 10^6 nucleotides, that is to say, about 10^4 adducts per cell. This is in the same general range as the detection limit of 5×10^4 molecules per cell reported for DNA-O^6-ethyldeoxy guanosine in liver sections of rats treated with diethylnitrosamine [22].

Thus the presently described simple and highly sensitive method for detecting carcinogen-DNA adducts in conventional histologic sections should prove useful for future investigation of metabolic activation and repair potential at the individual cell level. It would appear that generation of equivalent antibodies against other carcinogen-DNA adducts is a high priority for an understanding of the role played by such lesions in tumor induction.

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