Pyrimidine Dimer Removal Enhanced by DNA Repair Liposomes Reduces the Incidence of UV Skin Cancer in Mice

Daniel Yarosh,2 Lori Green Alas, Vivien Yee, Andrew Oberyszyn,3 Jeannie Tsimis Kibitel, David Mitchell,4 Rebecca Rosenstein, Alan Spinowitz, and Marc Citron

ABSTRACT

UV exposure has been linked to skin cancer in humans by epidemiology and the rare genetic disease xeroderma pigmentosum. However, UV produces multiple photoproducts in DNA, and their relative contribution is uncertain. An enzyme which specifically repairs cyclobutane pyrimidine dimers in DNA, T4 endonuclease V, was encapsulated in liposomes for topical delivery into mouse and human skin. In both species, liposomes applied after UV exposure localized in the epidermis and stimulated the removal of cyclobutane pyrimidine dimers. UV-irradiated mice treated with these liposomes had a dose-dependent decrease in the incidence of squamous cell carcinoma compared to controls. The results demonstrate that unrepaired cyclobutane pyrimidine dimers in DNA are a direct cause of cancer in mammalian skin.

INTRODUCTION

UV-induced carcinogenesis is important both as a model system for human cancer and for practical human health reasons: the etiological agent (UV light) is well defined, and its dosimetry can be experimentally controlled; few types of DNA lesions are produced; the target tissue (skin) is readily accessible, and the pathology of the malignant disease is defined; and most importantly, skin cancer is a very real disease for the rapidly growing number of people who develop it each year (1). UV produces two major genotoxic lesions in DNA, CPD, and (6–4) photoproducts, and several minor ones (2). In the human genetic disease xeroderma pigmentosum, patients have a biochemical defect in the DNA repair of both CPD and (6–4) photoproducts (3) and develop skin cancers on sun-exposed sites at greatly increased frequencies (4). Sorting out the relative contributions of these DNA lesions to human skin cancer has not been simple. Photoreactivation of CPD reduced cancer in the thymus of fish (5) and the skin of marsupials (6), but these animals are distantly related to humans, and photoreactivation of CPD also increased the removal of (6–4) photoproducts in frog cells (7). Mutations have been identified which originated in C-C dipyrimidines within the ras oncogene (8) and p53 tumor suppressor gene (9) in human skin cancers, but this provides only circumstantial evidence for the initial DNA lesion. Discrimination between these two major lesions can be accomplished by making use of the specificity of a DNA repair enzyme. T4 endonuclease V, the product of the phage T4 denV gene, initiates the removal of CPD from DNA by a lesion-specific glycosylase and apyrimidinic endonuclease activity (10). Purified T4 endonuclease V, encapsulated in liposomes and delivered to UV-irradiated cells in culture, increased CPD removal and DNA repair synthesis and enhanced survival in repair-deficient cells (11–13). The liposomes were more efficient than cell permeabilization or denV gene transfection (14).

Liposome encapsulation of drugs has been used for the topical application of agents to reduce systemic toxicity (15). We show here that liposome-encapsulated T4 endonuclease V penetrates and localizes in mouse and human skin, increases the specific removal of CPD from DNA, and reduces the incidence of skin cancer in UV-irradiated mice.

MATERIALS AND METHODS

T4N5 Liposomes. T4N5 liposomes were prepared by encapsulating purified T4 endonuclease V in liposomes composed of phosphatidyl choline, phosphatidyl ethanolamine, oleic acid, and cholesterol hemisuccinate (2:2:1:5 molar ratio) by the detergent dialysis method (12). The concentration of the entrapped enzyme was determined by the nicking of UV-supercoiled DNA with and without dissolution of the liposome (11). Control liposomes contained bovine (enzymatically inactive) T4 endonuclease V (12). For the topical treatment of mouse and human skin, the liposomes were mixed into a 1% hydrogel (Hysan SS201; Kingston Hydrogels, Dayton, NJ) made with phosphate-buffered saline. In the first photocarcinogenesis experiment, 5% dextran was substituted for 1% hydrogel. The liposome dosage was adjusted by the amount of liposomes added to the lotion.

Cyclobutane Pyrimidine Dimer and (6–4) Photoproduct Assay. The frequency of CPD in skin DNA was measured by the alkaline agarose gel assay (16). The epidermis was isolated by trypsin digestion of excised skin, and the DNA was purified by protease digestion, phenol extraction, and ethanol precipitation. The DNA was then treated with purified T4 endonuclease V to produce breaks at all CPD sites, and the single strands were separated by alkaline agarose gel electrophoresis. Photographic negatives of the ethidium bromide-stained gels were analyzed by computerized densitometry, which calculated the change in the average molecular length of DNA resulting from breaks at CPD sites. Mouse 10T1/2 fibroblasts were labeled with 14C]thymidine prior to UV irradiation and incubated for 48 hr in 4% fetal calf serum, and the removal of CPD and (6–4) photoproducts from UV-irradiated DNA was measured by radioimmunoassay (17).

Human Skin Organ Culture. Human breast skin was obtained immediately after surgery for mastectomy, cut into 1-cm2 pieces, and incubated dermal side down surrounded by Dulbecco's modified Eagle's medium with 10% fetal calf serum and 2 µg/ml hydrocortisone. The exposed epidermis was irradiated with UV-B from two FS20 sunlamp bulbs, calibrated with a UVX digital radiometer and UV31 probe (Ultra-Violet Products, San Gabriel, CA). The T4N5 liposome lotion was applied with a moist cotton swab, and after incubation the skin was fixed in 4% formaldehyde, paraffin embedded, and sectioned at 10 µm. The sections on slides were dewaxed, rehydrated, and incubated with antiendonuclease V polyclonal antibodies 14C-labeled by reductive methylation (18). The slides were then coated with nuclear track emulsion, exposed, developed, and photographed using a Nikon diaphot microscope at ×300.

Received 12/18/91; accepted 5/12/92.

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1 Supported in part by National Cancer Institute Small Business Innovative Research Grant 2R44-CA52401-01 to Applied Genetics Inc.
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5 The abbreviations used are: CPD, cyclobutane pyrimidine dimers; UV-B, ultraviolet B.

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Animal Care. SKH-1 hairless mice, 6–8 weeks old, were obtained from Charles River Laboratories (Wilmington, MA), housed in plastic cages with wire mesh covers, and fed standard Purina food pellets and water ad libitum, in compliance with the NIH Policy on Humane Care and Use of Laboratory Animals. The 12 h/day lighting was by fluorescent bulbs filtered with amber Toob-Gards (Malconite, Clifton, NJ) to eliminate photoreactivating light.

Skin Penetration and Histopathology. The animals were treated with T4N5 liposomes in 1% hydrogel lotion and then returned to Charles River Laboratories. There they were sacrificed, the dorsal skin was sectioned and stained, and the slides were interpreted by a veterinary pathologist who was unaware of the treatment. T4N5 liposomes were also prepared using [3H]oleic acid (10 Ci/mmol; New England Nuclear) and applied to mouse skin at 2 × 10⁸ dpm/ml. At various times after treatment the mice were sacrificed, and the treated skin was fixed, embedded in paraffin, and sectioned parallel to the dermal plane. The radioactivity in each section was measured by liquid scintillation counting.

Serum Chemistry. The animals were treated topically with T4N5 liposomes in 1% hydrogel 5 days/week for 3 weeks, or received one i.v. injection of T4N5 liposomes alone. The animals were sacrificed, and serum, separated by centrifugation, was sent to Tufts Veterinary Diagnostic Laboratory (N. Grafton, MA), for automated processing with a Hitachi 737 Chemistry Analyzer.

Systemic Distribution. T4 endonuclease V was reductively methylated with [14C]formaldehyde (56 mCi/mmol) and sodium cyanoborohydride and encapsulated in T4N5 liposomes (6 μg/ml, 4.7 × 10⁵ dpm/nmol, 2 × 10⁵ dpm/ml). The T4N5 liposomes were either administered topically or injected i.v., and at various times after treatment the animals were sacrificed, the blood was collected in heparin, and extracts were prepared from the vital organs by mincing and sonication. Samples of blood and organ extracts were assayed for radioactivity by liquid scintillation counting.

Photicarcinogenesis. SKH-1 mice were irradiated 3 times/week, unrestrained from above without cage covers, with two Westinghouse FS20 sunlamps (peak emission at 313 nm) calibrated by UVX digital radiometer and UV-31 sensor. Immediately afterward they were treated with active or inactive T4N5 liposome lotion on the dorsal skin with a moist cotton swab. Each week the animals were scored for skin lesion as described by Gallagher et al. (19) in the region of liposome application. After 30 weeks the animals were sacrificed, and lesions representative of each scoring group were examined histologically. Invariably lesions >1 mm in diameter were squamous cell carcinomas. Tumor incidence was calculated as the fraction of animals with at least one tumor >1 mm, and the mean time to first tumor was calculated from the Weibull survival distribution.

RESULTS

T4N5 Liposome Penetration and Localization in Skin. Application of T4N5 liposomes containing T4 endonuclease V to mammalian skin results in rapid penetration and localization in the epidermis. Normal human skin obtained within 1 h of mastectomy was cultured for 2 h and then treated topically with T4N5 liposomes in a 1% hydrogel lotion. Penetration was detected in skin sections by autoradiography using 14C-polyclonal antibodies to T4 endonuclease V (Fig. 1). Nonspecific binding was not observed in untreated skin (Fig. 1, A and B). Ten min after treatment, low levels of antibody binding were detected in the epidermis (Fig. 1, C and D), and by 1 h the binding of antibody in the epidermal layers is easily seen (Fig. 1, E and F).

Similar results were obtained using mouse skin treated with T4N5 liposomes and immunofluorescent microscopy, which also indicated that hair follicles were a significant pathway of skin penetration (data not shown). The liposomes remained localized in skin (Fig. 2).

Fig. 1. T4N5 liposome penetration of human skin. Human skin from mastectomy was left untreated (A and B) or treated topically with T4N5 liposomes at 1 μg/ml and incubated for 10 min (C and D) or 60 min (E and F). Penetration of the encapsulated T4 endonuclease V into the skin was detected by staining sections with 14C-antibodies and autoradiography.
each time point (each >1,000 dpm) were sectioned parallel to the epidermal plane, (O), 1 (•), 6 (A), and 24 (A) h posttreatment. Three treated skin samples from were treated topically with ([3H]oleic acid)-T4N5 liposomes and sacrificed at 0 and the radioactivity in each section was measured by scintillation counting. The percentage depth from the skin surface.

OrganBloodHeartKidneyLiverLungSpleenHour126126126126126126126126126126126126126126Topical0.0000150.000900.0120.00450.0170.0590.0920.0190.0370.00890.0073Intravenous3.12.63.22.70.34.55.46.79.91.54.28.910.714.12.92.410.516.24.38.88.08.217.48.42.03.97.38.89.30.7

Table 1 Systemic distribution of T4N5 liposomes

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Fig. 2. T4N5 liposome penetration of mouse skin. SKH-1 hairless female mice were treated topically with [3H]oleic acid-T4N5 liposomes and sacrificed at 0 (O), 1 (•), 6 (A), and 24 (A) h posttreatment. Three treated skin samples from each time point (each >1,000 dpm) were sectioned parallel to the epidermal plane, and the radioactivity in each section was measured by scintillation counting. The percentage of total radioactivity in each section was averaged and plotted against the percentage depth from the skin surface.

Enhanced Repair of Cyclobutane Pyrimidine Dimers. Topical treatment of UV-irradiated mouse or human skin with T4N5 liposomes does increase the removal of CPD (Fig. 3). Mice were UV-B irradiated and treated with liposomes encapsulating either active T4 endonuclease V or inactive (boiled) enzyme. After 6 h the CPD frequency in untreated controls was 78.0 ± 8.2 (SEM)/10⁶ bases. There were fewer CPD in the skin of mice treated with active liposomes compared to inactive controls. A similar pattern of enhanced CPD removal was seen after topical application to human skin organ cultures (Fig. 3). The shape of the dose-response curve was similar in mouse and human skin: increased CPD loss with increasing liposome dose up to about 60% of control CPD at 0.5 μg/ml, followed by no further repair enhancement at higher liposome concentrations. This dose-response curve is very similar to the curve of enhancement of unscheduled DNA synthesis in human explant keratinocytes treated with T4N5 liposomes (13).

In mouse cells the DNA repair enhanced by T4N5 liposomes is specific for CPD (Fig. 4). Mouse 10T½ fibroblasts in culture were UV irradiated and treated with T4N5 liposomes, and the loss of both CPD and 6-4 photoproducts was followed by radioimmunoassay. Few CPD were excised in untreated cells during the first 6 h, but T4N5 liposomes reduced the CPD frequency to 60% of controls. However, 6-4 photoproducts were rapidly removed from cellular DNA, and T4N5 liposomes had no effect on this rate.

Photocarcinogenesis. SKH-1 hairless mice have a low incidence of spontaneous skin cancer, and unirradiated animals treated for 30 weeks, 3 times/week, with 1 μg/ml T4N5 liposomes developed no tumors and had no weight change compared to untreated controls (data not shown). Two groups of 25 female mice each were irradiated 3 times/week with one minimal erythema dose of UV-B and immediately treated topically with either 1 μg/ml active T4N5 liposomes or inactive lotion. The incidence of tumors larger than 1 mm was reduced in the group treated with active liposomes compared to controls (Fig. 5A). The Weibull survival curves for the two groups were significantly different (P < 0.002), and the mean time to first tumor was increased from 19.5 weeks in the control group to 21.8 weeks in the active group (P < 0.01). At 30 weeks the

Enhanced Repair of Cyclobutane Pyrimidine Dimers. Topical treatment of UV-irradiated mouse or human skin with T4N5 liposomes results in penetration and localization in epidermis but no local inflammation and no systemic circulation or toxicity.
The yield of tumors was likewise reduced by 45% with T4N5 liposomes. This lower UV-B dose delayed the appearance of tumors in the control group from 19.5 weeks in the first experiment (Fig. 5A) to 23.8 weeks (Fig. 5B). Treatment with active T4N5 liposomes further reduced the incidence of tumors proportional to liposome dose (Fig. 5B). The mean time to first tumor in each group was calculated from the Weibull survival curve (Fig. 5, inset). Increasing concentrations of T4N5 liposomes increased the time to first tumor from 23.8 to 26.8 weeks (P < 0.002 by Tarone-Ware trend test). The shape of this dose-response curve is similar to that of the curve for CPD repair (Fig. 3) and unscheduled DNA synthesis in keratinocytes (13). The yield of tumors was likewise reduced by 45% with T4N5 liposome treatment, from 7.1/animal in the control group to 3.9/animal in the highest-dose group (P < 0.05).

In a discontinuous irradiation experiment, mice were irradiated with 1 minimal erythema dose of UV-B and treated afterward with T4N5 liposomes, 3 times/week. All irradiation was discontinued at 12 weeks, and liposome treatment was stopped after 14 weeks. At 25 weeks the animals treated with 0.5 μg/ml T4N5 liposomes had fewer tumors than controls treated with lotion alone (3.5 ± 0.8 versus 4.7 ± 0.7 tumors/mouse, respectively). This protocol produces a high variability relative to a low tumor yield, and the difference was marginally significant (P = 0.08 by the Mann-Whitney two-sample test).

**DISCUSSION**

Solar UV exposure has been clearly linked to human skin cancer, and the molecular biology of UV photocarcinogenesis is now being unraveled. Mutations activating ras oncogenes and inactivating p53 suppressor genes have been sequenced from human skin cancer DNA (8, 9). These occur most frequently at C-C sequences, which implicate UV-induced pyrimidine photoproducts by circumstance. The direct evidence for CPD as the initiating lesion relies on photoreactivation in non-mammalian systems which do not exclude increased repair of the 6-4 photoproduct (5-7).

Topically applied T4N5 liposomes penetrate human and mouse skin, enhance the removal of CPD, and reduce the incidence of skin cancer in mice. This is likely a true reduction and not a delay, since at equal tumor incidence liposome-treated groups always had fewer tumors per animal than controls. When treatment was discontinued before tumors arose, differences in tumor yield between treated and control groups still appeared.

The T4N5 liposomes localize in epidermis, produce no inflammation, and achieve little or no systemic circulation. Thus responses unrelated to DNA repair are not likely causes for the reduction in skin cancer. In cultured cells T4N5 liposomes enhance the repair of CPD with no effect on the repair of 6-4 photoproducts. Most importantly, the liposome dose-response curve is distinctive in reaching a plateau at the same liposome dose for CPD removal, DNA repair synthesis, and tumor incidence. This combination of biological localization, biochemical specificity, and concurrence of biochemical and biological dose-response provides strong evidence that the mechanism by which T4N5 liposomes reduce skin cancer is through the repair of CPD in DNA. The corollary of this argument is that CPD are one cause of cancer.

T4N5 liposomes may reduce cancer incidence by simply repairing CPD faster. A more intriguing possibility is that T4 endonuclease V gains access to regions, including nontranscribed sequences, which are not as easily repaired by the eukaryotic enzymes. CHO cells expressing T4 endonuclease V

Fig. 4. Repair of DNA damage in T4N5 liposome-treated mouse cells. Mouse 10T1/2 cells, irradiated with 10 J/m2 UV-C from a germicidal bulb, were untreated (•, □) or treated with 0.2 μg/ml T4N5 liposomes added to the cell culture media (○, △). At times after treatment the remaining CPD (○, □) and 6-4 photoproducts (△, △) were measured by radioimmunoassay.

The reduction of skin cancer was T4N5 liposome dose-dependent (Fig. 5B). Four groups of 20 animals each, both male and female, were irradiated 3 times/week with one-third the minimal erythemal dose of UV-B and immediately treated topically with either base lotion alone or increasing concentrations of T4N5 liposomes. This lower UV-B dose delayed the appearance of tumors in the control group from 19.5 weeks in the first experiment (Fig. 5A) to 23.8 weeks (Fig. 5B). Treatment with active T4N5 liposomes further reduced the incidence of tumors proportional to liposome dose (Fig. 5B). The mean time to first tumor in each group was calculated from the Weibull survival curve (Fig. 5, inset). Increasing concentrations of T4N5 liposomes increased the time to first tumor from 23.8 to 26.8 weeks (P < 0.002 by Tarone-Ware trend test). The shape of this dose-response curve is similar to that of the curve for CPD repair (Fig. 3) and unscheduled DNA synthesis in keratinocytes (13). The yield of tumors was likewise reduced by 45% with T4N5 liposome treatment, from 7.1/animal in the control group to 3.9/animal in the highest-dose group (P < 0.05).

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from the transfected denV gene removed CPD more quickly from both actively transcribed and noncoding DNA than did cells without the denV gene (21).

CPD do produce mutations at C-C dipyrimidines in human cells (22), and UV-induced mutations are more than likely an initiating event in photocarcinogenesis. However, UV produces other changes which influence the rate of skin cancer, in particular, suppression of the immune system (23). Suppressor cells arising in irradiated animals inhibit systemic immune responses (24), and this compromised immune surveillance fosters the tumorigenesis of initiated cells (25). Production of suppressor T-cells and other inhibiting immunomodulators may be triggered by CPD, since T4N5 liposomes are able to almost completely prevent the UV-induced suppression of systemic delayed-type hypersensitivity and contact hypersensitivity (26).

Thus CPD may cause cancer not only by serving as initiating lesions but also by stimulating responses which cause tumor promotion and progression. This may explain why the length of UV-B exposure as well as the total fluence is a controlling factor in tumorigenesis (27). The role of DNA damage in time-dependent events after initiation bears more careful examination.

T4 endonuclease V has been inserted into mammalian cells by DNA transfection, cell permeabilization, and microinjection, in each case enhancing DNA repair (reviewed in Ref. 28). Delivery by liposomes is more efficient for cells in culture than these methods (28), and the data presented here demonstrate that liposomes allow T4 endonuclease V to penetrate into living skin. The technology for producing and dermal application of liposomes has rapidly advanced (29), and T4N5 liposomes offer a practical method to reduce skin cancer in humans even after UV exposure.

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