Enhanced Unscheduled DNA Synthesis in UV-Irradiated Human Skin Explants Treated with T4N5 Liposomes

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Epidermal keratinocytes cultured from explants of skin cancer patients, including biopsies from xeroderma pigmentosum patients, were ultraviolet light-irradiated and DNA repair synthesis was measured. Repair capacity was much lower in xeroderma pigmentosum patients than in normal patients. The extent of DNA repair replication did not decline with the age of the normal patient. Treatment with T4N5 liposomes containing a DNA repair enzyme enhanced repair synthesis in both normal and xeroderma pigmentosum keratinocytes in an irradiation- and liposome-dose dependent manner. These results provide no evidence that aging people or skin cancer patients are predisposed to cutaneous malignancy by a DNA repair deficiency, but do demonstrate that T4N5 liposomes enhance DNA repair in the keratinocytes of the susceptible xeroderma pigmentosum and skin cancer population. J Invest Dermatol 97:147–150, 1991

The rate of skin cancer among whites has increased dramatically in recent years, not only in the U.S. but worldwide [1,2]. The interplay between human genes and response to solar ultraviolet light (UV) leading to skin cancer is not well characterized. Skin cancer rates increase with increasing age [1], but it is not known if this is due to an accumulation of damage or a decline in defense against UV exposure. In the extreme case of the hereditary disease xeroderma pigmentosum (XP), patients have a biochemical block in repair of DNA damage and have an enormous predisposition to skin cancer on sun-exposed skin [3].

The unscheduled DNA synthesis assay (UDS) is a widely used measure of DNA repair capacity because it measures the step of resynthesis of excised DNA using radiolabeled DNA precursors. UDS is able to identify fibroblasts from the classic XP complementation groups by their deficit in post-UV UDS compared to normal fibroblasts [4]. Recently it has been demonstrated that cultured epidermal keratinocytes from both the classic and variant forms of XP show reduced UDS compared to normals [5,6]. Indeed, evidence is accumulating that the defect in the variant form of XP includes a block in incision of UV-irradiated DNA. [7,8].

The biochemical deficiency in XP cells can be overcome by the introduction of DNA-damage-specific endonucleases into permeabilized cells [9,10]. The pyrimidine-dimer specific T4 endonuclease V (endo V) was encapsulated into liposomes and used to stimulate DNA repair in both UV-irradiated XP fibroblasts and normal human epidermal keratinocytes [11]. We report here on the DNA repair capacity of epidermal keratinocytes from skin cancer patients including XP patients, and the enhancement of repair in cells from these patients after treatment with T4N5 liposomes encapsulating endo V.

MATERIALS AND METHODS

Skin Explants and Keratinocyte Culture Two lots of normal human epidermal keratinocytes were purchased from Clonetics Corp., San Diego, CA. Human skin explants were prepared from uninvolved excess skin excised from patients during the normal course of Mohs surgery for basal and squamous cell carcinoma. All patients consented to the use of the skin in this research. In addition, 4-mm punch biopsies were collected from unexposed skin of consenting patients with clinically confirmed XP, and sent overnight in sterile media. The samples were trimmed of fat, cut into 1-mm² pieces, and dried dermal side down onto plastic dishes. The samples were fed with Dulbecco's modified Eagle's media with 10% fetal calf serum and 2 μg/ml hydrocortisone and incubated at 37°C in a 5% CO², humidified atmosphere. After 2 d, when halos of keratinocytes were first forming around the explants, feeder layers of mitomycin C-inactivated mouse 3T3 cells were added at 10⁵/ml in KCM media (50% Dulbecco's and 50% Ham's F-12 media, 5% fetal calf serum, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 30 μg/ml adenine, 10 ng/ml epidermal growth factor, 10 ng/ml cholera enterotoxin). After 7 d the halos of keratinocytes were 10–20 cells thick.

Normal human fibroblasts were cultured from skin explants by mincing the skin sample, digesting with 0.25% trypsin for 90 min at 37°C, and plating the dissociated cells in Dulbecco's media with 10% newborn calf serum. Colonies of fibroblasts appeared in 14 d.

Unscheduled DNA Synthesis The fibroblasts were plated alone whereas the keratinocytes were plated with inactivated feeder layers on glass slips. After overnight incubation, the cells were washed and irradiated with UV-C from a germicidal lamp (predominantly 254 nm) as monitored by a UVX digital radiometer. The cells were refed with media and ³H-thymidine (10 μCi/ml, 63 Ci/mmol). T4N5 liposomes were prepared and tested as described [12]. The lipid bilayer, composed of phosphatidyl choline, phosphatidyl ethanola mine, cholesterol hemisuccinate, and oleic acid, encapsulates active recombinant T4 endonuclease V at an internal concentration

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of 1 mg/ml. The concentration of T4N5 liposomes added to the cells after irradiation reflect the final enzyme concentration in the cell culture media.

After 4 h, the radioactivity and liposomes were removed and the cells fed with cold thymidine for an additional hour. The cells were then fixed with ethanol/acetic acid (3:1 v/v), washed extensively with 5% trichloroacetic acid and 70% ethanol, coated with Kodak NTB nuclear track emulsion, and exposed for 7 d. After development, the grains over the nuclei of 25 lightly labeled cells were counted, and averaged for each slip. The value of grains/nucleus at each UV dose was corrected for grains/nucleus in the absence of UV. The Student two-tailed t test was applied to calculate p values and determine statistical significance.

**Immunofluorescence** After the halo of keratinocytes formed around the skin explant, the cells were fixed with methanol and blocked with 10% goat serum. The cells were stained with monoclonal antibody AE1 (Boehringer Mannheim) specific for acidic epidermal keratins in the basal layer of skin [13]. Antibody binding was visualized with goat anti-mouse IgG linked to fluorescein isothiocyanate and a Nikon Diaphot epifluorescence microscope with a B2-A filter.

**RESULTS**

Explant keratinocyte cultures were established from clinically normal skin of ten patients (6 male, 4 female) who had Mohs surgery for removal of cutaneous neoplasms and from non-sun exposed skin of seven XP patients (four male, three female). Two days after plating the skin in plastic dishes, keratinocytes began to grow out from the edges of the sample, and these cells were used after 7 d in culture. At this point the cells expressed acidic lower molecular weight keratins characteristic of keratinocytes in the basal layer of skin (Fig 1). Keratinocytes grew from explants of XP patients as efficiently as explants from normal skin cancer patients. Two additional secondary keratinocyte cultures were obtained commercially; these were derived from clinically normal women. Fibroblasts were also grown from the skin of six normal skin cancer patients (four male, two female).

The DNA repair capacity of each keratinocyte or fibroblast culture was measured after 25 J/m² of UV-C by the UDS assay and plotted against the age of the patient (Fig 2). The mean grains/nucleus for keratinocytes and fibroblasts from normal patients differed by very little (7.62 ± 0.55 versus 7.86 ± 0.81) and they appear together in the figure. In only one case were both keratinocytes and fibroblasts cultured from the same sample; in this 67-year-old man the grains/nucleus for keratinocytes was 7.04 and for fibroblasts was 5.4. All samples were derived from patients with skin cancer, with the exception of two commercially obtained keratinocyte cultures, noted by superscripts 1 and 2 in Fig 2.

The ages of the donor patients ranged from 22 to 89 years. However, the DNA repair capacity varied little with age; the slope of the regression line relating grains/nucleus to age was -0.009 (fit = 0.788). This means that repair capacity declined by less than 13% (1/7.7 grains/nucleus) for every 100 years of life.

Keratinocytes from XP patients were deficient in DNA repair measured by UDS. Their average was 2.57 ± 0.83 grains/nucleus, or more than two standard deviations (SD) below the average for keratinocytes from normal skin cancer patients. The XP patients ranged in age from 2 to 18 years, and six of seven had had cutaneous neoplasms. None of the XP patients had been assigned to complementation groups. The repair capacity of individual XP patients varied greatly; the patients with the two highest UDS measurements were the youngest, at 2 and 3 years old. In one case the XP keratinocytes showed fewer grains/nucleus after UV irradiation than in unirradiated cells and therefore a value below zero appears in Fig 2. This patient was the most severely affected, with short stature, mental retardation, and central nervous system dysfunction.

The youngest normal skin cancer patient (36 years old with distinctive freckling and basal cell carcinoma on the eyelid, superscript 3 in Fig 2) showed a level of UDS nearly two SD less than the mean for all normal keratinocytes and within the range of the XP samples. After 10 J/m² UV-C these keratinocytes showed almost no detectable UDS (data not shown). A biopsy from this patient was sent to Dr. James Cleaver, Laboratory of Radiobiology at the University of California, San Francisco, for XP diagnostic testing. The results were that this patient's fibroblasts were not hypersensitive to UV-induced cell killing. However, they showed only 80% of normal post-UV incision activity, and the possibility that this patient was of the XP variant type was not excluded.

After UV irradiation with 10 and 25 J/m², T4N5 liposomes encapsulating the DNA repair enzyme endo V were added to the keratinocytes and fibroblasts during the UDS assay (Fig 3). Normal human keratinocytes treated with T4N5 liposomes showed a small increase in the background number of grains/nucleus in the absence of UV, most noticeably at 1 µg/ml liposomes. After irradiation with either 10 or 25 J/m², treatment with T4N5 liposomes at concentrations of 0.1, 0.2, or 1 µg/ml significantly enhanced UDS by a factor of two or more (p < 0.05). Figure 3 also shows that treatment of UV-irradiated skin fibroblasts with T4N5 liposomes either at 0.1 or 1 µg/ml significantly enhanced UDS (p < 0.05). In the absence of UV, T4N5 liposomes had an insignificant effect on UDS in fibroblasts.

The UDS response of UV-irradiated keratinocytes and fibroblasts as a function of T4N5 liposomes concentration is shown in Fig 4. DNA repair is initially stimulated by low concentrations of the liposome-encapsulated DNA repair enzyme after either 10 or 25 J/m², but the effect is saturated at liposome concentrations of be-
between 0.1 and 0.2 \(\mu g/\text{ml}\). In the presence of excess T4N5 liposomes the maximum level of UDS is determined by the UV fluence.

Treatment of XP keratinocytes with T4N5 liposomes increased the amount of DNA repair (Fig 5) by a factor of more than two \((p < 0.05)\) and in a dose-dependent manner, again reaching a plateau at about 0.1 \(\mu g/\text{ml}\). The UDS levels achieved in liposome-treated XP keratinocytes were within the range of repair capacity in normal keratinocytes.

**DISCUSSION**

Skin cancer is a major public health concern and T4N5 liposomes offer the potential of a unique preventive therapy by repairing DNA damage in epidermal keratinocytes after UV exposure but before the damage is fixed as oncogenic lesions. Among those who might benefit greatly from this therapy are XP patients, who must overcome a biochemical defect, and patients who have already developed a cutaneous malignancy and would benefit from faster repair of DNA lesions. The results here demonstrate that T4N5 liposomes enhance DNA repair in epidermal keratinocytes from these patients. The development of a topical lipidosome therapy must include studies on skin penetration (manuscript in preparation).

The explant keratinocyte culture method permits DNA repair measurements in keratinocytes grown from human skin after only a few days' culture. The keratinocytes represent cells from the basal, less-differentiated, layer. The DNA repair response of skin fibroblasts closely resembles the response of these explant keratinocytes.

Keratinocytes from the skin cancer patients did not show a marked deficiency in UV-stimulated UDS compared to the keratinocyte cultures from unaffected individuals. However, the DNA repair capacity of the youngest skin cancer patient was well below the norm. Lymphocytes from patients with actinic keratosis, a premalignant lesion, have reduced DNA repair synthesis [14], and patients with basal cell carcinoma show reduced excision of pyrimidine dimers from DNA compared to normals [15]. Thus, some portion of the skin cancer incidence may be attributable to DNA repair-deficient individuals, but a large sampling will be required to distinguish them from interindividual variation. UV-induced UDS did not significantly decline with age, and thus there is no evidence that the exponential increase in skin cancer incidence with age is related to a decline in DNA repair capacity.

**Figure 2.** UDS in human keratinocytes and fibroblasts after 25 J/m² UV-C irradiation as a function of donor age. Data from skin cancer and XP patients, except for symbols with superscripts 1 and 2, from unafflicted women. See Results for discussion of superscript 3. Line drawn through data from normal patients by linear regression analysis.

**Figure 3.** UDS in normal human keratinocytes and fibroblasts treated with T4N5 liposomes as a function of UV-C fluence. \textit{Error bars}, SEM. Where \textit{error bars} are not visible, SE was smaller than the symbol size.

**Figure 4.** UDS in normal human keratinocytes and fibroblasts as a function of T4N5 liposome dose. Each symbol represents an independent measurement using one explant. \textit{Error bars}, SEM of each measure. Where \textit{error bars} are not visible, standard error was smaller than the symbol size.

**Figure 5.** UDS in XP keratinocytes treated with T4N5 liposomes as a function of UV-C fluence. \textit{Error bars}, SEM.
Keratinocytes from XP patients ranged from marginally to severely deficient in UDS after UV exposure, consistent with the range of UDS response among the XP complementation groups [16]. None of the XP patients reported here have been assigned to complementation groups. The patient with the lowest capacity had the most severe physical and mental difficulties. A consistent finding is that patients with the lowest DNA repair abilities most frequently have neurologic abnormalities [17].

T4N5 liposomes are lipid spheres encapsulating the DNA repair enzyme endo V, which accomplishes the first, and rate-limiting, step of excision repair by incising the DNA strand containing the damage. Subsequent steps are performed by the endogenous exonucleases, polymerases, and ligases of the cell. T4N5 liposomes are shown here to stimulate DNA repair synthesis not only in repair-deficient XP keratinocytes, but also in normal keratinocytes and fibroblasts. In the case of XP keratinocytes, lipidome treatment increased their UDS levels to normal, and this may be understating the amount of repair because repair patches formed after endo V incision are smaller than normal [18], unpublished results. The enhancement in repair correlated with increasing liposome dose up to about 0.2 µg/ml, at which point a plateau (determined by the UV fluence) was reached. This suggests that in the presence of excess T4N5 liposomes incision is no longer the rate-limiting step and complete repair is accomplished faster.

Endo V has been introduced into XP fibroblasts by Sendai virus permeabilization resulting in stimulation of DNA repair [9,10]. T4N5 liposomes are more efficient than the permeabilization method because the enzyme is delivered in concentrated quanta; UDS was stimulated in XP cells after treatment with 0.05 µg/ml enzyme in liposomes, whereas permeabilization with Sendai virus required 1.5 µg/ml and reached a plateau at 34 µg/ml endo V [10]. Most importantly, T4N5 liposomes offer a potentially practical method of topical delivery of DNA repair enzymes to prevent skin cancer.

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