Topical Treatment with Liposomes Containing T4 Endonuclease V Protects Human Skin *In Vivo* from Ultraviolet-Induced Upregulation of Interleukin-10 and Tumor Necrosis Factor-α

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Exposing human skin to ultraviolet radiation causes DNA damage, sunburn, immune alterations, and eventually, skin cancer. We wished to determine whether liposomes containing a DNA repair enzyme could prevent any of the acute effects of irradiation when applied after ultraviolet exposure. Fifteen human patients with a prior history of skin cancer were exposed to two minimal erythema doses of ultraviolet radiation on their buttock skin. Liposomes containing T4 endonuclease V or heat-inactivated enzyme were applied immediately and at 2, 4, and 5 h after ultraviolet irradiation. Transmission electron microscopy after anti-T4 endonuclease V-staining and immunogold labeling on biopsies taken at 6 h after ultraviolet exposure revealed that the enzyme was present within cells in the skin. Immunohistochemical DNA damage studies suggested a trend toward improved DNA repair at the active T4 endonuclease V liposome-treated test sites. Although the active T4 endonuclease V liposomes did not significantly affect the ultraviolet-induced erythema response and microscopic sunburn cell formation, they nearly completely prevented ultraviolet-induced upregulation of interleukin-10 and tumor necrosis factor-α RNA message and of interleukin-10 protein. These studies demonstrate that liposomes can be used for topical intracellular delivery of small proteins to human skin and suggest that liposomes containing DNA repair enzymes may provide a new avenue for photoprotection against some forms of ultraviolet-induced skin damage. ***Key words: cyclobutane pyrimidine dimers/cytokines/DNA damage/DNA repair enzyme/photoprotection. J Invest Dermatol 114:149–156, 2000***

Exposure to solar ultraviolet (UV) radiation is the main environmental factor linked to the formation of skin cancers, which are the most common cancers in humans (Silberberg and Lubera, 1988). Wavelengths particularly in the UVB (280–320 nm) range damage DNA in the cells of human skin, and unrepaired UV-induced lesions in DNA, such as cyclobutane pyrimidine dimers (CPD), can give rise to mutations involved in skin tumorigenesis (Ananthaswamy and Pierceall, 1990). The importance of an effective DNA repair mechanism in inhibiting skin cancer development is best illustrated by the genetic disease xeroderma pigmentosum (XP), in which a defect in DNA repair is associated with the development of multiple skin cancers early in life, at a rate several thousand-fold greater than that in the normal population (Cleaver and Bootsma, 1975; Kraemer *et al*, 1994). In addition, UV-induced DNA damage is the initiating event for some forms of UV-induced immune suppression (Applegate *et al*, 1989; Kripke *et al*, 1992; Wolf *et al*, 1995), which contributes to skin cancer formation in rodents (Kripke and Fisher, 1976; Fisher and Kripke, 1982) and probably also in humans (Streilein *et al*, 1994a, b).

In the normal population, overloading DNA repair mechanisms by excessive sunlight exposure and a low DNA repair capacity may also be potential risk factors for the development of skin cancer (Lambert *et al*, 1976; Munch-Petersen *et al*, 1985; Thielmann *et al*, 1987; Alcalay *et al*, 1990; Wei *et al*, 1994, 1995; Grossman and Wei, 1995). Particularly, Wei *et al* (1994, 1995) have recently reported epidemiologic evidence comparing basal cell carcinoma patients with normal subjects that impaired DNA repair may be a susceptibility factor for sunlight-induced skin cancer in the general population.

In the cascade of events that occur as a consequence of DNA damage, the production of immunomodulatory cytokines from

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Abbreviations: CPD, cyclobutane pyrimidine dimer; CTTD, cyclobutane-type thymine dimer; SBC, sunburn cell; T4N5 liposomes, T4 endonuclease V-containing liposomes; XP, xeroderma pigmentosum.

keratinocytes and other target cells in the skin plays a crucial role in UV-induced immune suppression (Vermeer and Streilein, 1990; Rivas and Ullrich, 1992; Nishigori *et al*, 1996). Tumor necrosis factor (TNF)-α and interleukin (IL)-10 are involved in UV-induced suppression of contact allergy and delayed-type hypersensitivity responses, respectively (Vermeer and Streilein, 1990; Yoshikawa and Streilein, 1990; Rivas and Ullrich, 1992; Nishigori *et al*, 1996). Both cytokines have been shown to be upregulated in keratinocytes in response to UV-induced DNA damage. This conclusion was based on studies in which liposomes containing a DNA repair enzyme [T4 endonuclease V (T4N5 liposomes)] were used to reduce CPD and cytokine formation in culture and in murine skin *in vivo* (Nishigori *et al*, 1996; Kibitel *et al*, 1998). Thus, liposomes containing DNA repair enzymes potentially represent a novel approach for protecting against the harmful effects of sunlight exposure that result from UV-induced DNA damage. In mice, application of T4N5 liposomes to UV-irradiated skin protects against UV-induced suppression of contact allergy responses and delayed-type hypersensitivity responses to certain antigens (Kripke *et al*, 1992; Wolf *et al*, 1993b, 1995), and reduces the formation of skin cancers (Yarosh *et al*, 1992; Bito *et al*, 1995). In this study, we show in skin cancer patients, who may benefit more than normal subjects from topical DNA repair enzyme treatment, that T4N5 liposomes applied after UV exposure penetrate human skin and deliver T4 endonuclease into keratinocytes and epidermal Langerhans cells. Importantly, we demonstrate that T4N5 liposome treatment prevents UV-induced upregulation of the immunosuppressive cytokines IL-10 and TNF-α, under circumstances in which the UV-induced erythema (i.e., sunburn reaction) remains unaffected.

MATERIALS AND METHODS

Patients and clinical study design The clinical study protocol was approved by the Ethical Committee of the Karl Franzens University, Graz, Austria. Fifteen patients with a history of at least one skin cancer were enrolled in the study after giving their informed consent. The mean age of the patients (two women and 13 men) was 63.5 y (range 36–79 y). The skin type of the patients according to Fitzpatrick's classification was as follows: eight, skin type I; four, skin type II; and three, skin type III. Six patients had a history of basal cell carcinoma, five of squamous cell carcinoma, and four of both. Nine of the 15 patients had had multiple skin cancers, eight additional solar keratoses, and three of them also cutaneous malignant melanoma. At the time of this study, all patients were free of skin cancer and solar keratosis.

Two sets of test material, consisting of active and inactive liposome lotion, each with a coded label A and B, were prepared by Applied Genetics Inc. Dermatics (Freeport, NY) for each patient. Each patient was randomly assigned a study number which corresponded to the code designating which buttock side received the active T4N5 or inactive T4N5 liposome lotion. Each patient received two individual minimal erythema doses (MED) of UV radiation through a template to four sites of 2×2 cm diameter each, two on the left buttock (sites 1 and 2) and two on the right buttock (sites 3 and 4), located at least 2 cm above the site of MED testing. The remaining skin was shielded from the light source. Immediately after UV exposure, the test lotion A was applied liberally (40–100 μl per cm^2) by a cotton swab moistened with saline to the UV-irradiated site 2 and the test lotion B was applied by a new moistened cotton swab to the UV-irradiated site 3. Within 10 min after UV exposure, a 4 mm punch biopsy was taken under local anesthesia with 1% lidocaine hydrochloride from the UV-irradiated site 4 that had been left untreated. At 2, 4, and 5 h after UV exposure we reapplied the coded test liposome preparations. At 6 h post-UV exposure, 4 mm punch biopsies were obtained under local anesthesia from the three remaining UV-irradiated test sites (1–3) and from an unirradiated and liposome-untreated area (site 5) on the left side. All biopsies were sliced into four 1 mm strips before fixation and/or further processing.

T4N5 liposomes T4N5 liposomes were prepared by encapsulating purified recombinant T4 endonuclease V in liposomes composed of phosphatidylcholine, phosphatidylethanolamine, oleic acid, and cholesteryl hemisuccinate (2:2:1:5 molar ratio). The enzyme in the liposomes was quantified by enzyme-linked immunosorbent assay and assayed for activity using UV-irradiated DNA as a substrate (Yarosh *et al*, 1992). The T4N5 liposomes were suspended in a lotion which consisted of 1% Hypan SS 201 hydrogel (Hymedix International Inc. Dayton, NJ) in phosphate buffered saline at a concentration of 1 μg per ml of T4 endonuclease V encapsulated in liposomes. The placebo liposome preparation was chemically identical except that T4 endonuclease V was inactivated by boiling before encapsulation.

UV source UV radiation was provided by a high-pressure metal halide lamp Sellasol 1200 (Sellas Medizinische Geräte GmbH, Gevelsberg, Germany), which has its lower cut-off at 293 nm and delivers 5.5% of its total energy within the UVB (280–320 nm) and 94.5% within the UVA (320–400 nm) wavelength range. According to the CIE action spectrum for UV-induced erythema in human skin, 95.5% of the relative cumulative erythemal effectiveness of a Sellasol 1200 lamp results from its UVB and 4.5% from its UVA portion of the emission spectrum. The mean UVB irradiance at 30 cm distance from the light source was 0.56 mW assuming monochromatic light at 297 nm, as determined by an IL 1700 radiometer equipped with an SED 240 detector fitted with an NS 297 interference filter and a W5352 quartz diffuser (International Light, Newburyport, MA).

Minimal erythema dose Each patient was tested by a standard UV irradiation protocol for the individual MED. Six 2×2 cm areas on the untanned buttocks were UV-irradiated with graded doses of UV at 40% increments. Erythema was scored 24 h after UV exposure and the MED was defined as the lowest dose required to produce erythema with a sharp border.

Reflectance spectroscopy Erythema and pigmentation at the test sites were monitored by reflectance spectroscopy (Diffey *et al*, 1984) using the DermaSpectrometer (Cortex Technology, Hadsund, Denmark). This handheld apparatus provides a read-out of a relative erythema/melanin-index, based on the absorbency characteristics of the human skin. The erythema/melanin-index was measured at all test sites before UV exposure and at 2, 4, and 6 h after exposure. At each time point, five separate measurements were performed per test site and the average of the results was used for data analysis.

Histology Specimens were fixed in 4% buffered formalin, processed routinely, sectioned at 4 μm, and stained with hematoxylin and eosin. The specimens were examined microscopically by the same observer (H.P.S.) in a blinded fashion for gross and specific histopathologic alterations. The presence of epidermal spongiosis, dermal edema, and dermal cellular infiltrate was rated on a scale from 0 to 4 as follows: 0, none; 1, minimal; 2, moderate; 3 medium; and 4, extensive. Sunburn cells (SBC) cells were defined as eosinophilic cells with pyknotic nuclei and were counted in the interfollicular epidermis in random high-power fields (400 ×). Thirty fields were examined per treatment site for the presence of SBC. Counts were expressed as the mean ± SEM number of SBC per centimeter length of epidermis.

Electron microscopy This was performed according to the procedure of Stossel *et al* (1990). Immunogold labeling was done by sequential incubation of grids with the following reagents: 50 μg per ml of affinity purified rabbit anti-T4 endonuclease V antibody (Applied Genetics Inc. Dermatics) for 2 h at room temperature, and 1:10 dilution of Auroprobe goat anti-rabbit antibody (15 nm gold) (Amersham, Arlington Heights, IL) for 1 h. After each step, the grids were washed with Tris-buffered saline, rinsed with distilled water several times, dried, and counter-stained with uranyl citrate and lead citrate in an LKB ultrastainer (Leica, Deerlake, IL). The samples were examined in a JEOL 1200-EX transmission electron microscope at 80 kV.

Immunohistochemical staining and automated image analysis of CTTD Tissue strips fixed for 24 h in 4% formalin and then transferred to a 70% ethanol solution were used for the CTTD (cyclobutane-type thymine dimer) assay using TDM-1 antibody (Mizuno *et al*, 1991) and the Discovery automated computerized image analysis system (Becton Dickinson, Leiden, The Netherlands), as previously described (Potten *et al*, 1993; Chadwick *et al*, 1995; Young *et al*, 1996). Immunohistochemical staining with TDM-1 antibody was performed on 3 μm thionine counter-stained sections. TDM-1 antibody has been found to be specific for cyclobutane-type thymine (TT) dimers and does not recognize cytosine containing dimers CC, CT, or TC (Mizuno *et al*, 1991). The amount of specific antibody binding was visualized by immunohistochemical microscopy, quantified by digitized image analysis, and expressed in terms of integrated mean optical density per cell (Potten *et al*, 1993). The nuclear staining in at least 200 representative nuclei was measured per specimen and biopsy site.

Immunohistochemical staining and automated image analysis of Langerhans cells Four micrometer cryostat sections from snap-frozen 1 mm thick tissue strips were immunohistochemically stained using a CD-1 mouse monoclonal antibody (OKT 6, Ortho Pharmaceutical, Raritan, NJ) and a three-step immunoperoxidase technique with amino-ethyl-carbazole as the chromogen. Counterstain was performed with Mayer's hematoxylin. Automated image analysis with minor modifications was done, as previously described (Smolle and Hofmann-Wellenhof, 1998). Image analysis was applied to 10–15 fields sampled from each specimen section of the different biopsy sites. The data were used to calculate the mean number of Langerhans cells bodies per mm epidermis length, percentage of CD-1-positive structures per epidermal section area and length of CD-1-positive structures (i.e., dendrites) per epidermal section length (μm per mm).

***In situ* hybridization for IL-10 and TNF-α mRNA** Formalin-fixed, paraffin-embedded skin biopsy samples were tested using a modification of the method described by Lan *et al* (1996). Briefly, after permeabilization with proteinase K and microwaving 5 μm tissue sections were hybridized with a digoxigenin-labeled 574 nt anti-sense riboprobe corresponding to nucleotides 344–917 of the human TNF-α cDNA and a 1000 nt riboprobe corresponding to nt 411–1410 of the human IL-10 cDNA, kindly provided by Dr. Mark W. MacEwen (University of Alabama, Birmingham, AL). Hybridization signal was detected with anti-digoxigenin, biotinylated antibody, and avidin–peroxidase conjugate developed with diaminobenzidine-H_2O_2. Positive cells in the entire epidermis were counted microscopically by a blinded observer (R.R.M.) under × 400 magnification in five to 10 fields per specimen and treatment site. Cytokine-positive cells were defined as cells with perinuclear ring-like granular staining. Hybridization with sense probes (to assess background staining) and application of hybridization buffer with no probe served as negative controls.

Immunohistochemical staining for IL-10 and TNF-α protein Deparaffinized tissue section (4 μm) were stained with either monoclonal mouse anti-human TNF-α antibody, polyclonal rabbit anti-human TNF-α antibody, or polyclonal mouse anti-human IL-10 antibody (Genzyme Diagnostics, Cambridge, MA) at different dilutions. Secondary antibody staining was performed on a TechMate Horizon™ automated stainer (LJL Biosystems, DAKO, Vienna, Austria) using DAKO ChemMate peroxidase/AEC rabbit/mouse detection kit K 5003 (DAKO). Sections were counter-stained with hematoxylin.

Statistical analysis Application of the test liposome lotions and all subsequent clinical and laboratory studies were performed in a blinded manner, and the code was broken after study completion to perform statistical analysis. The significance of differences among different treatment and biopsy sites for the different biologic end-points was determined using the Wilcoxon's test. The statistical difference in the amount of gold particles in the electron microscopy studies and in cytokine-positive cells in the *in situ* hybridization studies among the different treatment sites was evaluated using the unpaired Student's t test (two-tailed). A difference was considered to be statistically significant when $p \leq 0.05$.

RESULTS

Electron microscopy studies revealed T4 endonuclease V enzyme in both keratinocytes and Langerhans cells Detailed transmission electron microscopy (TEM) studies were performed on two patients (E.H. and L.W.) whose samples had the best ultrastructural morphology. All samples from sites treated with T4N5 liposomes showed gold-labeled anti-T4 endonuclease V antibody staining throughout the epidermis and dermis (**Fig 1*A***). Gold particles were observed in the cytoplasm and nuclei of keratinocytes and Langerhans cells (**Fig 1*B***). Some Langerhans cells had more label than the surrounding keratinocytes, which was associated with Birbeck granules (**Fig 1*C***). Gold particles were also seen in the intercellular spaces, and this pattern was quite prominent in the stratum corneum and granular layer of the epidermis. Gold particles were counted in four to 13 fields on prints taken at 5000-fold and enlarged to 13,000-fold magnification in the entire epidermis except the stratum corneum. The number of gold particles per field ranged from 64 to 306. The mean ± SEM number of particles per micron square was 0.42 ± 0.03 in the liposome-untreated site, 1.09 ± 0.07 in the active T4N5-liposome-treated site, and 0.37 ± 0.01 in the control liposome-treated site of patient E.H. The difference between the active T4N5-liposome-treated site and other treatment sites was statistically significant (unpaired Student's t test, $p < 0.0001$). Similar results were found in patient L.W. Liposomal structures (multilamellar vesicles) such as seen in the murine experiments (Yarosh *et al*, 1994) were not observed in this study except for occasional electron-lucent inclusion structures seen in macrophage-like cells with some labeling in the upper dermis.

Trend toward increased repair of DNA damage by T4N5 liposomes As shown in **Table I**, there was significant endogenous DNA repair in the untreated skin as measured by automated image analysis of antibody staining against CTTD. At 6 h after UV exposure, normal endogenous repair reduced the amount of CTTD by 10.9% (Wilcoxon's test, $p = 0.030$). Active T4N5 liposomes reduced the CTTD by 18.0% ($p = 0.015$) and inactive control liposomes reduced CTTD by 12.3% ($p = 0.021$) *versus* UV-irradiated, untreated time 0 sample. The difference in DNA repair between T4N5 liposome-treated and control liposome-treated sites did not reach statistical significance, although the p-values suggested a trend toward accelerated DNA repair by the topical application of the active T4N5 liposomes even at the early time of 6 h after UV exposure.

No effect of T4N5 liposomes on UV-induced erythema, pigmentation, and SBC formation As shown in **Fig 2**, there was a statistically significant increase of the erythema (**Fig 2*A***) and melanin (**Fig 2*B***) indices as measured by reflectance spectroscopy at 2, 4, and 6 h after UV exposure irrespective of the treatment; however, there was no statistically significant difference in the erythema and melanin index among the different UV-irradiated test sites. Histologic examination of hematoxylin and eosin-stained specimens revealed statistically significant SBC formation at 6 h after UV exposure, ranging from a mean ± SEM of 7.8 ± 1.6 to 10.0 ± 1.4 in the different UV-irradiated test sites *versus* 1.4 ± 0.5 SBC per cm epidermis length in nonirradiated skin (Wilcoxon's test, $p \leq 0.05$). There were no statistically significant differences, however, in the mean number of SBC among the different treatment sites at 6 h after UV exposure. There were also no statistically significant UV-induced increases in epidermal spongiosis, dermal edema, and dermal infiltrate among the different treatment sites (data not shown).

Moderate reduction of Langerhans cell number by T4N5 liposome treatment after UV exposure Immunohistochemical CD-1 staining and automated image analysis of epidermis revealed a small but significant reduction of Langerhans cells number 6 h after UV exposure to 2 MED and treatment with active T4N5 liposomes (**Table II**). The number of Langerhans cell bodies was reduced to 76% when compared with the unirradiated and liposome-untreated control site. Similarly, the percentage of CD-1-positive structures per epidermal section area and length of CD-1-positive structures (i.e., dendrites) per epidermal section length was slightly reduced 6 h after UV exposure and active T4N5 liposome treatment. There were no statistically significant differences, however, among the other biopsy sites for any Langerhans cell parameter.

T4N5 liposomes prevent UV-induced upregulation of IL-10 and TNF-α *In situ* hybridization studies on the biopsy samples from three randomly selected patients (H.K, M.A., and F.G.) revealed that UV irradiation resulted in significant upregulation of IL-10 and TNF-α mRNA in the epidermis at 6 h after UV exposure (**Figs 3** and **4**). IL-10 and TNF-α staining was mostly restricted to perikaryal areas of basal and suprabasal layer cells (**Fig 3*C*, *E***). Some positive cells, however, were found throughout the entire epidermis. The dermal areas of the sections did not show positive signals, except for IL-10 in one sample (UV-irradiated only site of patient F.G.). In this patient we noted 1.3 ± 0.7 IL-10-positive cells in the dermis per × 400 field. Control specimens with no probe gave consistently negative results for all biopsy specimens examined, and hybridization with sense probes affected only

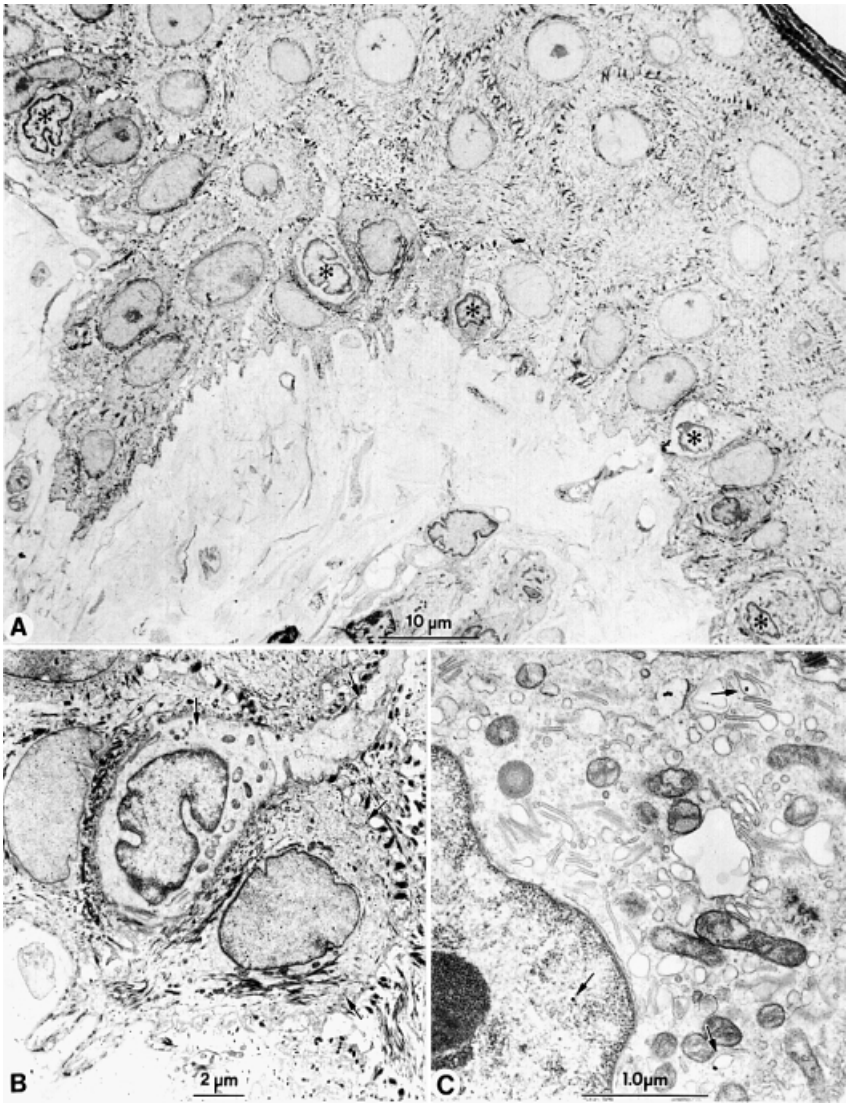

Figure 1. Electron microscopy studies indicate penetration of the T4N5 liposomes into the skin. TEM and gold-labeled anti-T4 endonuclease V staining of T4N5-liposome-treated skin biopsied 6 h after UV exposure. (*A*) TEM morphology of epidermis showing good ultrastructural detail and five Langerhans cells (*) at the basal layer. (*B*) Immunogold labeling showing gold label on Langerhans cells and keratinocytes (*arrows*). (*C*) High magnification micrograph of Langerhans cells showing gold label inside Birbeck granules and nucleus (arrows).

nonspecific background staining (data not shown). Quantitative analysis of cytokine-positive epidermal cells revealed that in all individual cases UV-induced mRNA upregulation of both IL-10 and TNF-α was statistically significant, compared with the unirradiated and untreated control site (Student's t test, $p < 0.02$) (**Fig 4**). In all cases examined there was a statistically significant protective effect of the active T4N5 liposomes (**Fig 3*D***) but not control liposomes (**Fig 3*E***) on UV-induced upregulation of IL-10 as well as TNF-α mRNA, compared with the UV-irradiated only control site at 6 h (Student's t test, $p < 0.02$).

UV exposure also resulted in upregulation of IL-10 protein expression at 6 h (**Fig 5**). The staining pattern indicated that the majority of cytokine-positive cells were keratinocytes. We also observed, however, staining of some cells in the dermis, including endothelial cells as well as fibroblasts and/or macrophages (**Fig 5*A*, *C***). Treatment with active T4N5 liposomes (**Fig 5*B***) but not inactive T4N5 liposomes (**Fig 5*C***) protected from IL-10 protein upregulation. In contrast to the finding for IL-10, we were unable to detect UV-induced upregulation of TNF-α protein at 6 h after UV exposure using monoclonal mouse anti-human TNF-α antibody or polyclonal rabbit anti-human TNF-α antibody over a broad range of antibody dilutions (data not shown).

Table I. Trend toward accelerated CTTD repair by T4N5 liposomes

UV exposure[a]	Treatment[b]	Time of biopsy[c]	CTTD (MOD ± SEM)[d]	CTTD removal[e]	p-value[f]
2 MED	None	0 h	438 ± 41	–	–
2 MED	None	6 h	392 ± 35	10.9	0.030
2 MED	T4N5	6 h	362 ± 35	18.0	0.015
2 MED	Hi-T4N5	6 h	386 ± 46	12.3	0.021
None	None	6 h	15 ± 4	–	–

[a]2 MED, exposure to two minimal erythema doses.
[b]T4N5, liposomes containing active T4 endonuclease V; HI-T4N5, liposomes containing heat-inactivated T4 endonuclease V.
[c]Time of biopsy after UV exposure.
[d]CTTD, cyclobutane-type thymine dimers as measured in mean ± SEM optical density (MOD).
[e]Percent CTTD removal compared with the UV-irradiated and untreated site at 0 h, corrected for background signaling at the unirradiated and liposome-untreated control site.
[f]p-value, as determined by Wilcoxon test when compared with UV-irradiated control site at 0 h; n = 15 subjects.

DISCUSSION

We found that exposure of normal human skin to UV radiation resulted 6 h later in significant upregulation of mRNA for the immunosuppressive cytokines IL-10 and TNF-α in the epidermis and that this upregulation was reduced by more than 90% by treatment with T4N5 liposomes. In addition, UV exposure also

upregulated IL-10 protein at 6 h and this effect was likewise prevented by active, but not inactive T4N5 liposomes. Similar to our observation, Enk *et al* (1995) have previously shown *in vitro* and *in vivo* that IL-10 gene expression in human keratinocytes is constitutive and can be upregulated upon UV exposure. In contrast to the finding for IL-10, we were unable to show UV-induced upregulation of TNF-α protein. This difference may be due to different kinetics of protein translation and turnover. Indeed, Strickland *et al* (1997) have recently reported that TNF-α mRNA and protein were constitutively expressed *in vivo* in unirradiated human skin and that UV-induced upregulation of this cytokine was minimal by 8 h and reached a maximum by 24 h after exposure. The immunoprotective capacity of the T4N5 liposomes found in this study is similar to that observed in a murine study, in which the T4N5 liposomes reduced UV-induced IL-10 protein expression in the epidermis as well as serum and prevented suppression of delayed-type hypersensitivity to alloantigen (Nishigori *et al*, 1996). Although this study did not allow the exact identification of cell types producing IL-10, the cytokine staining pattern indicated that the majority of IL-10-positive cells in the epidermis were keratinocytes and that there were also some IL-10-positive endothelial cells as well as fibroblasts and/or macrophages in the dermis (**Fig 5*A*, *C***). Indeed, previous studies have shown that human keratinocytes (Enk *et al*, 1995; Grewe *et al*, 1995, 1996; Kang *et al*, 1998), melanocytes (Teunissen *et al*, 1997), and macrophages (Kang *et al*, 1998) may be capable to produce IL-10 upon UV exposure.

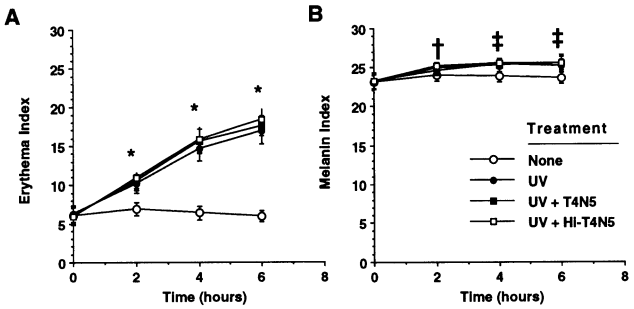

Figure 2. No effect of T4N5 liposomes on UV-induced erythema and pigmentation. Reflectance spectroscopy measurements of erythema and pigmentation after UV exposure. (*A*) Mean erythema and (*B*) melanin index ± SEM at the unirradiated and liposome-untreated (none), UV-irradiated and liposome-untreated (UV), UV-irradiated and active T4N5 liposome-treated (UV + T4N5), and UV-irradiated and heat-inactivated T4N5 liposome-treated (UV + Hi-T4N5) test site. Note there was statistically significant erythema at all time points after UV exposure irrespective of the treatment, *$p < 0.0002$ at 2, 4, and 6 h, as compared with the unirradiated and liposome-untreated control site (*A*). There was no statistically significant difference in erythema among the different UV-irradiated test sites. Note there was also a statistically significant increased melanin index at all time points after UV exposure irrespective of the treatment, †$p < 0.05$ at 2 h and ‡$p < 0.005$ at 4 and 6 h, as compared with the unirradiated and liposome-untreated control site (*B*). There was no statistically significant difference in melanin index among the different UV-irradiated test sites.

Our findings are important because at least two observations implicate immunosuppressive cytokines in human UV carcinogenesis: First, UV radiation is able to suppress the contact hypersensitivity induction in less than 40% of normal healthy subjects, but in over 90% of skin cancer patients (Yoshikawa *et al*, 1990). The UV-induced suppression of contact hypersensitivity has been linked to UV induction of TNF-α expression (Streilein *et al*, 1994a, b). Second, in the cancer predisposing disease XP, defective DNA repair leads to the increased release of soluble mediators (Yarosh *et al*, 1993), including TNF-α (Kibitel *et al*, 1998), and increased inhibition of intercellular adhesion molecule-1 (Krutmann *et al*, 1994). Importantly, the XP-A knockout mouse is more sensitive than normal littermates to UV-induced immune suppression (Miyauchi Hashimoto *et al*, 1996) and skin cancer (Nakane *et al*, 1995).

The TEM studies demonstrated that the T4 endonuclease V incorporated into the liposomes penetrated the skin and was successfully delivered into cells. The reduction in DNA damage (i.e., CTTD) in sites treated with active T4N5 liposomes was greater than that in control liposome-treated sites, but the difference at the 6 h time point only suggested a trend and did not reach statistical significance. This may be an underestimate because CTTD seem to be more slowly repaired than other forms of CPD (Niggli and Cerutti, 1983; Bykov *et al*, 1999) and the repair measured by the TDM-1 antibody is slower than that measured by other systems (Young *et al*, 1996; Bykov *et al*, 1999). Mitchell *et al* (1992), however, have recently reported an approximate 1:1 ratio for thymine (TT) *versus* cytosine containing (CC, CT, TC) dimers in UV-irradiated skin. Thus, CTTD seem to be nevertheless an appropriate and valuable end-point to measure DNA damage and repair. This study is consistent with previous findings that T4N5 liposomes enhanced DNA repair in XP patients using the same immunohistochemical method for detecting CTTD to nearly 20% at 6 h after UV exposure (Yarosh *et al*, 1996). Previous studies have also consistently found that repair of only a fraction of CPD can have a disproportionate biologic effect. For instance, Kripke *et al* (1992) reported that T4N5 liposomes nearly completely prevented UV-induced systemic immune suppression whereas they reduced overall DNA damage by only 50%. Nishigori *et al* (1996) showed *in vitro* in murine keratinocytes that T4N5 liposomes increased the amount of CPD removal by approximately 20% whereas they reduced UV-induced IL-10 protein production by up to 90%. Selective increases in DNA repair have dramatic effects on increased cell survival (Cleaver *et al*, 1995) and signal transduction

Table II. Moderate reduction of Langerhans cell number after UV exposure and T4N5 treatment

UV exposure[a]	Treatment[b]	Time of biopsy[c]	No. of Langerhans cells[d]	Percentage Langerhans cells per area[e]	Total Langerhans cells dendrite length[f]
2 MED	none	0 h	27.4 ± 2.2 (86)	1.85 ± 0.26 (101)	1477 ± 151 (102)
2 MED	none	6 h	31.3 ± 1.6 (98)	1.85 ± 0.19 (101)	1256 ± 127 (87)
2 MED	T4N5	6 h	24.4 ± 2.0 (76)[g]	1.54 ± 0.18 (84)	1240 ± 104 (86)
2 MED	Hi-T4N5	6 h	29.5 ± 2.4 (92)	1.76 ± 0.27 (96)	1351 ± 178 (93)
none	none	6 h	31.9 ± 1.8 (100)	1.83 ± 0.18 (100)	1450 ± 162 (100)

[a]2 MED, exposure to 2 minimal erythema doses.
[b]T4N5, liposomes containing active T4 endonuclease V; HI-T4N5, liposomes containing heat-inactivated T4 endonuclease V.
[c]Time of biopsy after UV exposure.
[d]No. of Langerhans cells, mean ± SEM number of Langerhans cells bodies per mm epidermis length.
[e]Percentage Langerhans cells per area, mean ± SEM percentage of epidermal area occupied by Langerhans cells.
[f]Total Langerhans cells dendrite length, mean ± SEM total length of Langerhans cells dendrites in mm per mm epidermis length.
[g]At 6 h after UV exposure the number of Langerhans cells bodies was significantly reduced at the active T4N5-treated site when compared with the unirradiated and untreated control site (Wilcoxon test, $p = 0.016$). The numbers in parentheses represent percentages as compared with the unirradiated and untreated control site for the different Langerhans cells parameters; $n = 15$ subjects.

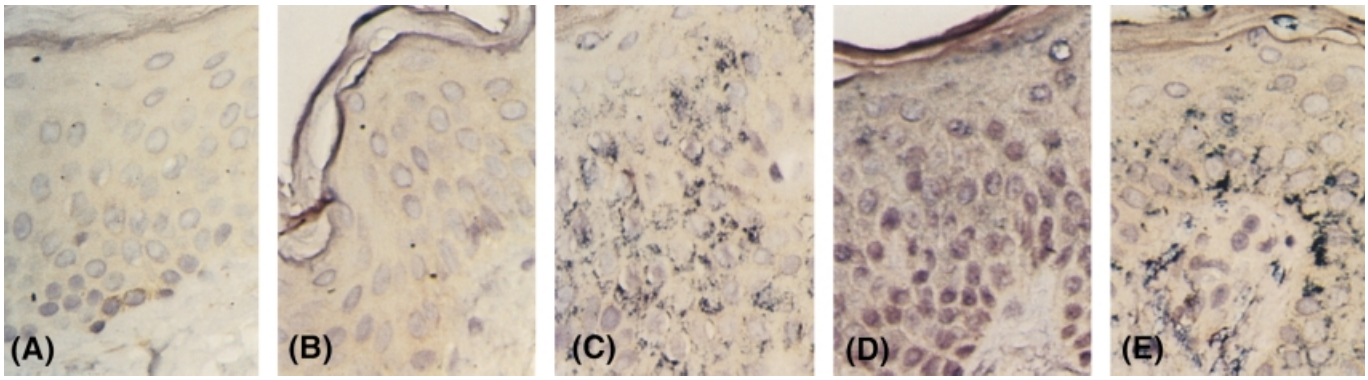

Figure 3. Qualitative protection by T4N5 liposomes against UV-induced upregulation of IL-10 mRNA expression. *In situ* hybridization studies for IL-10 mRNA expression in patient F.G. (*A*) Normal unirradiated site; (*B*) UV-irradiated and liposome-untreated site, biopsy taken shortly (10 min) after UV exposure; (*C*) UV-irradiated and liposome-untreated site; (*D*) UV-irradiated and active T4N5 liposome-treated site; (*E*) UV-irradiated and heat-inactivated T4N5 liposome-treated site, biopsies taken 6 h after UV exposure. Note UV exposure resulted within 6 h in upregulation of IL-10 mRNA message (granular staining) (*C*). IL-10 mRNA expression was mostly restricted to perikaryal areas of basal and suprabasal layer epidermal cells. Treatment with active T4N5 liposomes (*D*) but not heat-inactivated T4N5 liposomes (*E*) protected from IL-10 mRNA upregulation.

(Blattner *et al*, 1998). For example, the enhanced repair of actively transcribed genes by T4N5 liposomes may selectively alter gene expression (G. McGregor, personal communication).

This study agrees with previous findings that at early times, e.g., 2, 4, 6, and 24 h after UV, T4N5 has little or no effect on erythema (Yarosh *et al*, 1996). In XP patients at 48 h, however, T4N5 liposome lotion did decrease the erythema sensitivity (Yarosh *et al*, 1996). In murine studies (Wolf *et al*, 1993b, 1995), T4N5 liposomes only marginally reduced the sunburn reaction as measured by skin edema but clearly reduced or even completely prevented other UV-induced immunobiologic effects, including suppression of DTH to *Candida albicans* and alterations of Langerhans cells. The inability of T4N5 liposomes to protect against the UV-induced sunburn reaction is in contrast to studies in the South American opossum *Monodelphis domestica*, in which photoreactivation DNA repair protected against a variety of UV-induced biologic effects, including erythema (Ley, 1985) and edema (Applegate *et al*, 1985). This difference from this study, however, may be due to differences in the kinetics of the T4 endonuclease V-stimulated excision repair (relatively slow) (Yarosh *et al*, 1992) and the photoreactivation repair (more rapid) (Applegate *et al*, 1989). Nevertheless our observations underline the concept that T4N5 liposomes may be effective in preventing DNA damage and other associated effects even after an inflammatory sunburn response has been initiated by UV radiation.

Gilchrest *et al* (1993) recently reported that T4N5 liposome treatment enhanced UV-induced melanogenesis in both murine melanoma cells and human melanocytes. The T4N5 liposome-enhanced pigmentation, however, i.e., melanogenesis, increased between 16 and 96 h, and the most significant increase was seen at the late time points. The early time point of 6 h in this study may have prevented us from observing a similar effect. Indeed, the pigmentation observed in this study between 2 and 6 h after UV exposure must have been rather immediate and/or persistent pigment darkening due to photooxidation of melanin than true pigmentation. In a previous murine study, T4N5 liposomes had reduced the number of SBC at 24 h after UV exposure by approximately 50% (Wolf *et al*, 1995). In this study, the application of T4N5 liposomes did not significantly affect UV-induced SBC at 6 h after UV irradiation. SBC at this early time may be due to direct activation of the CD95 receptor (Aragane *et al*, 1998) and the SBC found at 24 h are more likely to reflect apoptosis induced by DNA damage (Ziegler *et al*, 1994). We also attempted to measure by computerized automated image analysis the effects of UV exposure and T4N5 liposomes on Langerhans cells in the skin (**Table II**). We were, however, unable to detect any morphologic changes or reduction in the number of Langerhans cells in the skin after UV exposure alone, which may be also due to the early 6 h time point of examination. The reason for the small reduction in the number of Langerhans cells at the active T4N5-liposome-treated site after UV exposure remains unknown at present.

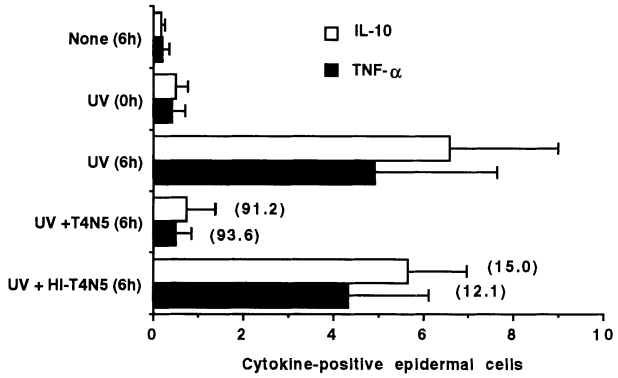

Figure 4. Quantitative protection by T4N5 liposomes against UV-induced upregulation of IL-10 and TNF-a mRNA expression. Quantitative analysis of *in situ* hybridization studies for IL-10 and TNF-α mRNA expression. The data shown represent the mean ± SEM number of cytokine-positive epidermal cells per field at 400 × magnification for the different treatment sites from three patients: None (6 h), unirradiated and liposome-untreated site; UV (0 h), UV-irradiated and liposome-untreated site at 10 min; UV (6 h), UV-irradiated and liposome-untreated site at 6 h; UV + T4N5 (6 h), UV-irradiated and active T4N5 liposome-treated site at 6 h; and UV-HI-T4N5 (6 h), UV-irradiated and heat-inactivated T4N5 liposome-treated site at 6 h after UV exposure. Values in parentheses represent the mean percent protection from UV-induced upregulation of cytokine mRNA message. The percent protection was determined by the following formula $[1-(A-B)/(C-B)] \times 100$, where the letters represent the average cytokine-positive epidermal cells in (*A*) liposome-treated and UV-irradiated site, (*B*) unirradiated and liposome-untreated site, and (*C*) UV-irradiated and liposome-untreated site at 6 h.

Taken together, the results of this study demonstrate that liposomal delivery represents an effective way of introducing proteins into the cells of human skin, including keratinocytes and Langerhans cells. Our results show that a DNA repair enzyme delivered in this manner can reverse some of the deleterious effects of UV irradiation that seem to be caused by DNA damage, such as the upregulation of the immunosuppressive cytokines, IL-10 and TNF-α. Topical DNA repair enzyme application therefore may be a clinically useful approach of photoprotection in humans. In contrast to conventional sunscreens, which are effective due to their content of chemical and/or physical UV filters, liposomes containing DNA repair enzymes may be able to protect against UV-induced damage to the skin, even when they are applied after UV exposure and initiation of the sunburn reaction. Thus, the

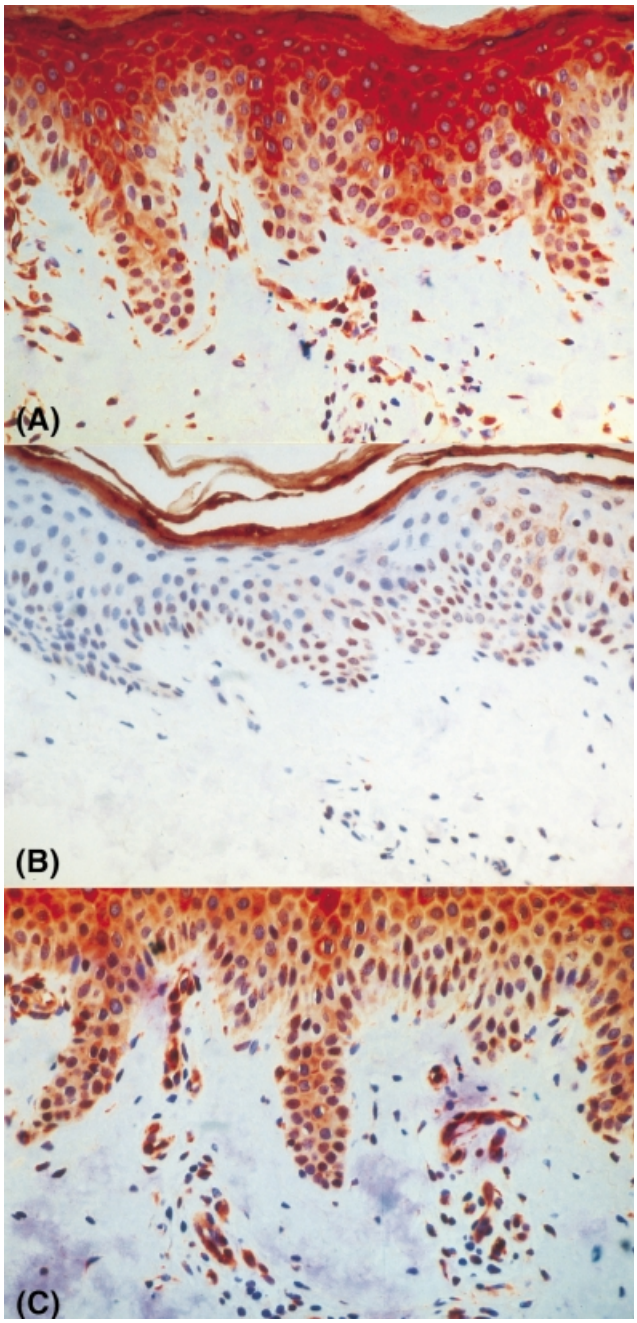

Figure 5. T4N5 liposomes prevent UV-induced upregulation of IL-10 protein. Immunohistochemical studies for IL-10 protein in patient F.G. (*A*) UV-irradiated and liposome-untreated site; (*B*) UV-irradiated and active T4N5 liposome-treated site; (*C*) UV-irradiated and heat-inactivated T4N5 liposome-treated site, biopsies taken 6 h after UV exposure. Note UV exposure resulted within 6 h in upregulation of IL-10 protein (red–brown staining) (*A*). Protein expression was detected in the entire epidermis but was most prominent in the suprabasal and granular cell layers. Treatment with active T4N5 liposomes (*B*), but not heat-inactivated T4N5 liposomes (*C*) protected from IL-10 protein upregulation. There was none to very weak protein expression in normal unirradiated skin and in UV-irradiated, liposome-untreated skin biopsied 10 min after exposure (data not shown). The primary antibody used for immunohistochemical staining was a polyclonal mouse anti-human IL-10 antibody at a dilution of 1:20.

immunoprotective effects of topical DNA repair enzyme application may open new avenues for photoprotection, particularly by protecting efficiently against the effects of UV radiation on the immune system, which are not always prevented by sunscreening agents (Wolf *et al*, 1993a,b, 1994; Granstein, 1995).

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