

# Green Tea Polyphenol Treatment to Human Skin Prevents Formation of Ultraviolet Light B-induced Pyrimidine Dimers in DNA<sup>1</sup>

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## ABSTRACT

Cancer chemopreventive effects of polyphenols from green tea (GTP) in mouse models of photocarcinogenesis are established. The present study is extended from mouse model to human system *in vivo* to determine the effect of topical application of GTP to human individuals against UV light-induced DNA damage in the form of cyclobutane pyrimidine dimers (CPDs) in the skin. UVB-induced CPDs were detected by immunohistochemical technique using monoclonal antibodies to thymine dimers. With the gradual increase in UVB dose, both erythema response and CPD formation in the skin was increased. GTP treatment inhibited both UVB-induced erythema response as well as CPD formation. Topical treatment with GTP (~1 mg/cm<sup>2</sup> of skin area) 20 min before human buttock skin (sun-protected site) exposure to UVB inhibited CPD formation in epidermis by 81, 70, 60, and 60% at 0.5, 1.0, 2.0, and 4.0 minimal erythema dose of UV exposure, respectively. Treatment of human skin with varying doses of GTP (1–4 mg/2.5 cm<sup>2</sup> of skin area) before a single dose of UVB exposure (4.0 minimal erythema dose) decreased dose dependently the formation of UVB-induced CPDs in both epidermis and dermis. The inhibition of UVB-induced CPDs by GTP treatment may be, at least in part, responsible for the inhibition of photocarcinogenesis. Our data suggest that GTP may be used as a novel chemopreventive candidate and possible strategy to reduce UV-induced skin cancer risk in the human population.

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## INTRODUCTION

Green tea is consumed worldwide as a beverage. In recent years, many studies have suggested that green tea possesses anti-inflammatory and anticarcinogenic effects (1, 2–5). The most convincing evidence for the anticarcinogenic effects of green tea came from studies in mouse skin tumor model systems of chemical and photocarcinogenesis. We and others have demonstrated that treatment with GTP, a polyphenolic fraction isolated from green tea, or its major constituent, (–)-epigallocatechin-3-gallate, topically or through drinking water to mice before UV<sup>3</sup> irradiation of dorsal skin prevents UVB-induced immune suppression (6–8) and photocarcinogenesis (9–13). In this report we describe a novel property of GTP, in that we show that topical treatment of human skin with GTP before UVB exposure prevents UVB-induced DNA damage measured in the form of CPD formation in skin target cells. It has long been documented that photochemical damage induced by UV light in chromosomal DNA of skin cells, predominantly in the form of CPDs, plays an important role in immune suppression (14, 15) and skin cancer induction (14, 16–18). CPDs were detected by immunohistochemistry technique using monoclonal antibodies to thymine dimers. Furthermore, because UV light-induced skin cancer risk is largely associated with Caucasians, in this study we focused our attention only on the Caucasian population.

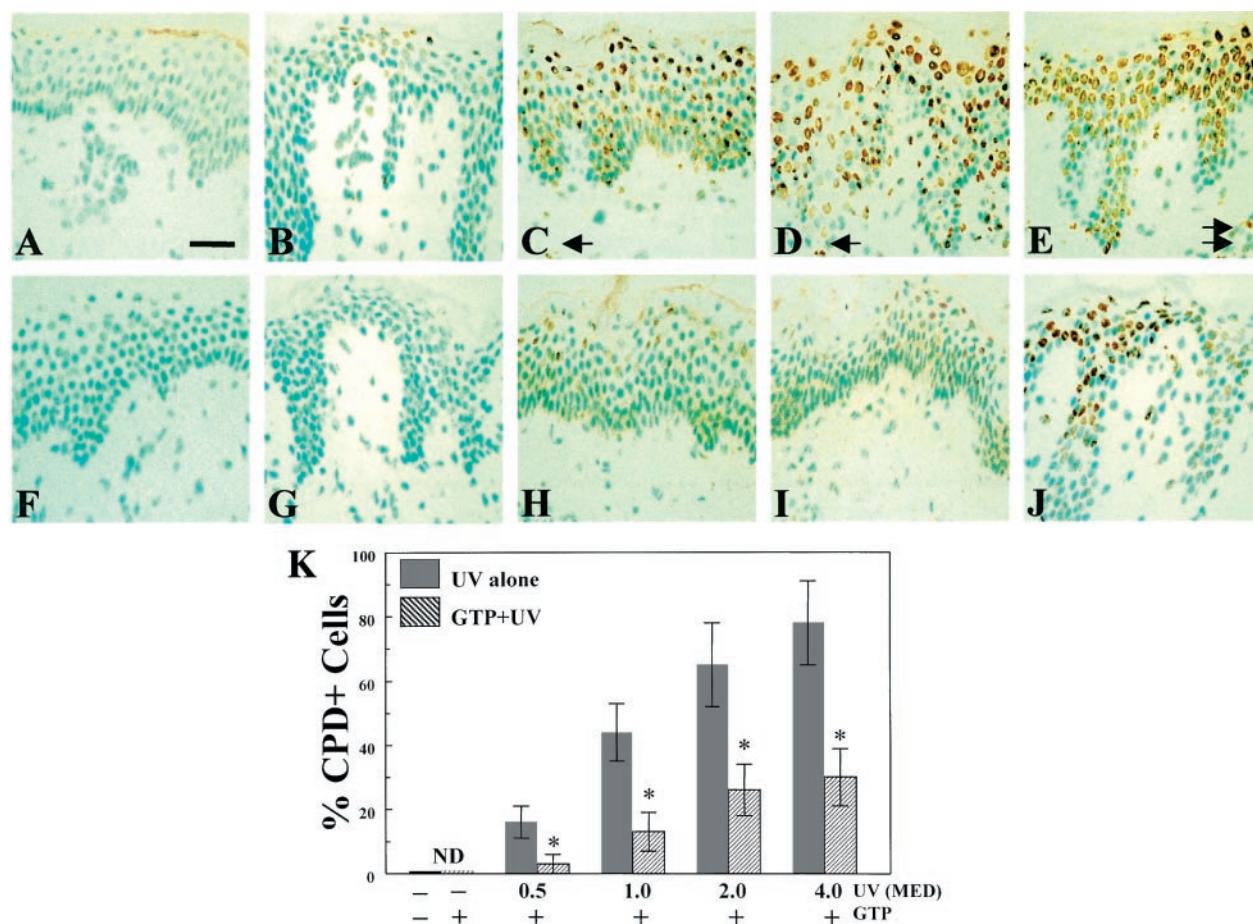
## MATERIALS AND METHODS

**Chemicals, Antibodies, and Reagents.** Monoclonal antibody to cyclobutane thymine dimers (clone KTM53, IgG1) was purchased from Kamiya Biomedical Company (Seattle, WA). Biotin-conjugated goat anti-mouse IgG1 was purchased from Caltag Laboratories, Inc. (San Francisco, CA). Diaminobenzidine reagent set and peroxidase-labeled streptavidin were purchased from Kirkgard and Perry (Gaithersburg, MD).

GTP (95% pure) was purchased from Natural Resources and Products International, Inc. (Englewood, NJ). GTP is known to contain four major polyphenolic epicatechin derivatives, namely epicatechin (6%), epigallocatechin (5%), epigallocatechin-3-gallate (65%) and epicatechin-3-gallate (24%; Ref. 19).

**Human Subjects and Skin Punch Biopsies.** Experiments with human subjects were performed in accordance with the approved institutional protocol. All individuals voluntarily participated in the study and gave written informed consent, which was approved by the Institutional Review Board of Uni-

<sup>3</sup> The abbreviations used are: GTP, a polyphenolic fraction isolated from green tea; UV, ultraviolet; UVB, ultraviolet B light; CPD, cyclobutane pyrimidine dimer; CPD+, CPD-positive; MED, minimal erythema dose.



**Fig. 1** Topical treatment with GTP (3 mg/2.5 cm<sup>2</sup>) to human skin inhibits UVB dose-dependent induction of CPD formation. Immunostaining was performed to detect UVB-induced DNA damage in the form of CPD+ cells 24 h post-UV exposure as described in "Materials and Methods." CPD+ cells are shown in dark brown. CPD+ cells were not detectable in non-UVB exposed (A) and non-UVB-exposed but GTP-treated (F) control skin. UVB-induced CPD+ cells are shown at 0.5 MED (B), 1.0 MED (C), 2.0 MED (D), and 4.0 MED (E) of UV doses 24 h post-UV exposure, whereas the protective effect of GTP is shown after 0.5 MED (G), 1.0 MED (H), 2.0 MED (I), and 4.0 MED (K) of UV exposures. Representative sections of immunostaining are shown from six different individuals with similar results. Scale bar = 100  $\mu$ m. B, topical treatment with GTP (3 mg/2.5 cm<sup>2</sup>) to human skin decreased the number of UVB-induced CPD+ cells. Increasing doses of UV exposure to skin increased CPD+ cells in the skin. In non-UV-exposed skin, CPD+ cells were not detectable. The number of CPD+ cells in epidermis after immunostaining was counted under the microscope using an ocular micrometer grid as described in "Materials and Methods." The number of CPD+ cells are represented as the percentage CPD+ cells  $\pm$  SD 24 h post-UV exposure from six different individuals with similar results. ND, not detectable. \*, statistically significant versus UV alone;  $P < 0.0005$ .

versity Hospitals of Cleveland, Cleveland, OH. Six Caucasian human subjects, both male and female, ranging from 25 to 55 years old, were recruited for this study. All subjects were in good health with no evidence of acute or chronic disease. Subjects were excluded if they were pregnant or nursing, had a history of an abnormal response to sunlight, or were taking a medication with known photosensitizing properties. On the first visit, the MED of UV was determined on buttock skin sites for each recruited individual using Westinghouse FS20 bulbs. The percentage of wavelengths emitted from these bulbs in the UVA, UVB, and UVC regions are 43%, 54%, and 3%, respectively (20). To determine each individual's MED, five to eight skin sites (1 cm  $\times$  2 cm) in a row were used and exposed to UV radiation with gradually increasing exposure times in increments of 30 s. On the second visit, 24 h later, the UV-exposed

sites were examined, and the lowest UV dose that induced a minimally perceptible redness at the exposure site was identified as the MED. On the volunteers' third visit, a baseline chromameter (Minolta) reading was taken and the buttock skin sites (sun-protected) were exposed to the desired dose of UV irradiation with or without pretreatment with GTP. Volunteers returned 24 h later (fourth visit) and exposed skin sites were visually examined for erythema formation, and chromameter readings were taken to express UV-induced redness in terms of the erythema index. After measuring erythema, skin punch biopsies 4.0 mm in diameter and 0.8 mm deep were taken from all skin sites from each individual and snap-frozen immediately after removal in OCT liquid embedding medium under liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further use.

To determine the effect of GTP on CPD formation induced

by different doses of UVB, different skin sites were exposed to 0.5, 1.0, 2.0, or 4.0 MED of UVB irradiation. GTP (3 mg/skin site/50  $\mu$ l of acetone) was topically applied 20 min before UVB exposure to determine its effect on UVB-induced CPD formation. On one skin site, GTP alone (non-UVB exposed) was applied to determine its effect, if any, on the CPD formation. In control skin sites (without any treatment, and non-UVB exposed) and UVB-alone-exposed skin sites, a 50- $\mu$ l vehicle (acetone) was topically applied to maintain similar treatment regimen in each group of individuals. Skin punch biopsies were obtained 24 h after UVB exposure.

For studies on GTP dose-dependent response on UV-induced CPD formation, different doses of GTP (1–4 mg/skin site/50  $\mu$ l acetone) were topically applied 20 min before exposure to 4 MED of UV. The skin site that was exposed to UV alone served as a positive control, whereas non-UV exposed skin site was used as a negative control. Thus, six skin punch biopsies were obtained from each individual 24 h after UV exposure.

**Immunostaining of CPDs.** To detect UV-induced CPD+ cells, immunostaining of CPDs was performed using the procedure described previously (21). Briefly, 6- $\mu$ m-thick frozen skin sections were thawed, and slides were kept in freshly prepared 70 mM NaOH in 70% ethanol for 2 min to denature nuclear DNA followed by neutralization for 1 min in 100 mM Tris-HCl (pH 7.5) in 70% ethanol. Slides were then washed once in 70% ethanol and twice in PBS for 5 min each. After washing, the slides were incubated for 30 min in 10% goat serum in PBS to prevent non-specific antibody binding. Sections were then incubated with thymine dimer-specific monoclonal antibody or its isotype control IgG1. Bound anti-CPD antibody was detected by incubation with biotinylated goat anti-mouse IgG1, followed by peroxidase-labeled streptavidin. Slides were developed with diaminobenzidine as a substrate for 6–7 min. The sections were then rinsed with distilled water and counterstained with methyl green (1% for 60 min), cleared, and mounted. The diaminobenzidine-peroxidase reaction gave a brown reaction product, and the methyl green gave a blue nuclear counterstain.

**Analysis of CPD+ Cells.** To determine the inhibitory effect of GTP on UVB-induced CPD formation, CPD+ cells in the epidermis and dermis compartments of the stained skin sections were counted at six to eight places using an ocular micrometer grid with  $\times 200$  magnification under a Zeiss Axiophot microscope and Zeiss Plan-Neofluar objective. Ocular micrometer grid corresponds to 0.0625 mm<sup>2</sup>. After counting the number of CPD+ cells in three sections per individual specimen, the number of CPD+ cells in each treatment group were expressed as a percentage  $\pm$  SD of the mean count from at least six different individuals. Non-UV irradiated normal control skin and GTP-alone-treated skin did not show CPD+ cells.

**Microscopy and Photography.** Images from immunostaining experiments were obtained using a Zeiss Axiophot microscope (Thornwood, NY) and Kodak Ektachrome 160T film (Rochester, NJ). These images were scanned (SprintScan software, Cambridge, MA) and formatted as *tiff* images in Adobe Photoshop. Composite figures were made using Microsoft Powerpoint software.

**Statistical Analysis.** All experiments were performed in six different individuals. The results were expressed as the mean number of CPDs  $\pm$  SD. Statistical analysis of all data between UV exposure alone and GTP-treated, plus UV-exposed groups, were determined by Student's *t* test. A *P* < 0.05 was considered statistically significant.

## RESULTS

**Treatment with GTP Inhibits UVB Dose-dependent Induction of CPD Formation in the Skin.** Photochemical damage to DNA is considered an important factor in the development of skin cancer. As shown in Fig. 1, UV exposure of human skin induces a dose-dependent increase in thymine dimers in nuclear DNA in epidermis as well as in dermis. CPD+ cells were not detectable in non-UV-exposed human skin, but even a sub-erythral dose of UV exposure (0.5 MED) to skin resulted in detectable levels of cells that contained pyrimidine dimers. Most of the CPDs were detected in epidermal cells, whereas comparatively lower numbers of CPDs were easily detectable in the dermis. In dermis, these dimers were observed both in papillary as well as in reticular dermis, as shown in Fig. 1A. Quantitative analysis of CPD+ cells under the microscope indicates an increasing number of CPD+ cells in epidermis with the increase in UV dose, as shown in Fig. 1B. The number of epidermal CPD+ cells in UVB-alone-exposed sites were  $16 \pm 5$ ,  $44 \pm 9$ ,  $65 \pm 13$ , and  $78 \pm 13$ ; whereas GTP-treated and then UVB-exposed sites contained  $3 \pm 3$ ,  $13 \pm 6$ ,  $26 \pm 8$ , and  $31 \pm 9$  at 0.5, 1.0, 2.0, and 4.0 MED of UVB exposure, respectively. Thus, pretreatment with GTP significantly inhibited UVB-induced CPD formation at each dose of UV exposure. The percentage of inhibition of CPD by GTP treatment at 0.5, 1.0, 2.0, and 4.0 MED of UV exposures was 81%, 72%, 60%, and 60%, respectively, which is highly significant (*P* < 0.0005).

**Treatment with GTP Inhibits UVB-induced Erythema Response in the Skin.** UVB-induced erythema in skin is considered a marker of tissue injury and inflammation. In our

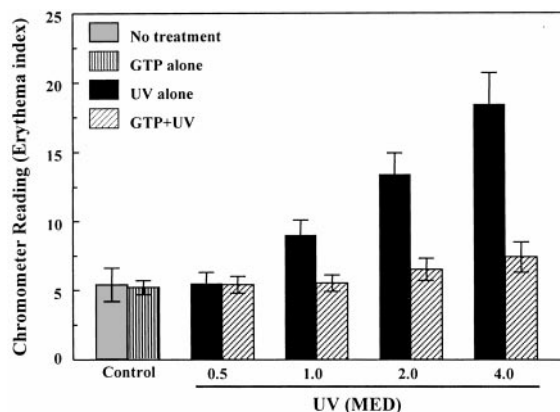
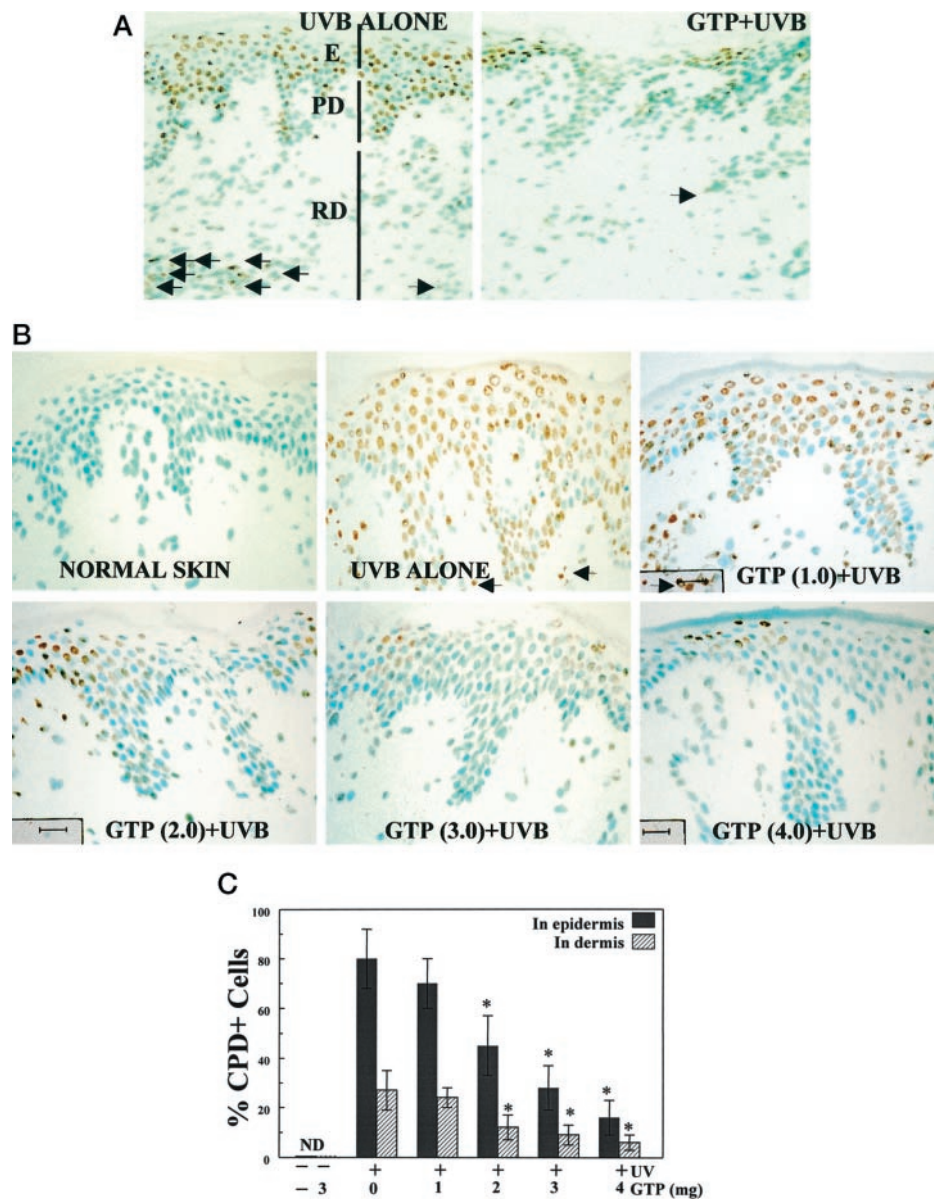


Fig. 2 Topical treatment with GTP before UVB exposure to human skin inhibits UVB-induced erythema response. UVB doses (0.5–4.0 MED) were delivered onto the specific skin sites. Twenty-four h after UVB exposure, the intensity of red color was measured using chromameter and referred as erythema index to compare the redness among other treated groups. The control skin sites were not UVB-irradiated. Erythema index is represented as mean  $\pm$  SD from six different individuals.





**Fig. 3** A, topical treatment with GTP before UVB exposure to human skin inhibits the penetrating depth of UV-light into the skin. Inhibition of penetration of UV light is detected by the presence of UV-induced CPD formation. *Arrows heads* show the CPD+ cells in deeper dermis. Representative sections of immunostaining are shown from six different individuals with similar results. *E*, epidermis; *PD*, papillary dermis; *RD*, reticular dermis. B, topical treatment with GTP before UVB exposure dose-dependently inhibits UVB-induced CPD formation in human skin. Representative sections showing GTP dose-dependent (1–4 mg/2.5 cm<sup>2</sup> skin area) inhibition of UVB (4 MED)-induced DNA damage in the form of CPD formation in the skin. Immunostaining was performed to detect UV-induced DNA damage in the form of CPD+ cells as described in “Materials and Methods.” CPD+ cells are shown as dark brown. CPD+ cells are not detectable in normal (non-UV exposed) or GTP-alone-treated (non-UV exposed) skin sites. UV-induced CPD+ cellular staining is shown 24 h post UV exposure with or without GTP treatment. Representative sections of immunostaining are shown from six different individuals with similar results. Scale bar = 75  $\mu$ m. C, treatment of GTP before UVB exposure dose-dependently inhibits UVB (4 MED)-induced CPD formation in epidermis and dermis 24 h post UV exposure. In non-UV exposed skin CPD+ cells are not detectable. The number of CPD+ cells after immunostaining in different treatment groups was counted under the microscope using an ocular micrometer grid with  $\times 200$  magnification as described in “Materials and Methods.” The number of CPD+ cells are represented as a percentage of CPD+ cells  $\pm$  SD in epidermis and dermis in different treatment groups from six individuals with similar results. ND, not detectable. \*, statistically significant versus UVB alone;  $P < 0.0005$ .

study, we found that UVB exposure to skin dose-dependently induced erythema development when measured 24 h after UV exposure. Topical treatment with GTP (3 mg/2.5 cm<sup>2</sup> of skin area) before UVB exposures (0.5–4.0 MED) inhibited signifi-

cantly UVB-induced erythema response as accounted for 100, 97, 86, and 84% respectively at 0.5, 1.0, 2.0 and 4.0 MED of UVB exposure, as shown in Fig. 2. GTP treatment alone did not affect the normal skin in terms of erythema appearance.

**Treatment with GTP Inhibits UV-induced CPD Formation in the Deeper Dermis.** As can be seen by the data in Fig. 3A (left panel), a number of CPD+ cells were clearly visible in the deeper reticular dermis (arrow heads). The detection of UV-induced CPD+ cells by immunohistochemistry demonstrates that a significant fraction of UV radiation penetrates across the dermal layers. It appears that treatment with GTP inhibits the penetrating capability of UV radiation into the deeper dermis, which results in the significant decrease in DNA damage in the form of CPD+ cells in the dermis, particularly in the reticular dermis compartment (Fig. 3A, right panel).

**Treatment with GTP Dose-dependently Inhibits UV-induced CPD Formation in the Skin.** Various doses of GTP ranging from 1 to 4 mg/2.5 cm<sup>2</sup> of skin area were topically applied to skin sites 20 min before UV (4 MED) exposure. As shown by immunohistochemistry (Fig. 3B), the treatment dose of 1 mg GTP/2.5 cm<sup>2</sup> of skin area did not effectively inhibit UVB-induced CPD formation, but significant inhibition in UV-induced CPD formation was observed at 2-, 3-, and 4-mg doses of GTP. Quantitation of CPD+ cells in each treatment group indicates that GTP treatment before UV exposure dose-dependently inhibited UV-induced CPD formation in the epidermis as well as in the dermis. The number of CPD+ cells in each treatment site is expressed in as a percentage of CPD+ cells, as shown in Fig. 3C. GTP treatment was found to inhibit UV-induced CPD formation by 12, 44, 65, and 80% in the epidermis at the treatment doses of 1, 2, 3, and 4 mg/skin site, respectively. Similarly, GTP treatment inhibits UV-induced CPD formation by 11, 62, 66, and 78% in dermis at the doses of 1, 2, 3, and 4 mg/skin site, respectively. Thus, except for the 1-mg dose of GTP treatment, highly significant ( $P < 0.0005$ ) inhibition by GTP was observed against UV-induced DNA damage in both epidermis and dermis. These results clearly demonstrate that GTP from green tea has remarkable potential to inhibit the UV-induced DNA damaging effects in the skin cells.

## DISCUSSION

DNA damage by UV radiation plays an essential part, and is the initial step, in skin cancer induction (16, 17). In the present study, we demonstrate the protective effect of GTP against UV-induced DNA damage. UV damage of cell nuclei could be observed both in epidermis and dermis. The outer layers of epidermis were more heavily damaged than the deeper layers. We found that treatment with GTP to human skin before UV exposure inhibits UV-induced DNA damage when detected by using immunohistochemistry. Our data also shows that GTP treatment inhibits (partially) the penetrating ability of UV radiation into the deeper dermis (Fig. 3A), thus protecting against DNA damage in dermal cells as well. These results are correlated with prior observations where GTP treatment to mouse skin was found to protect against UVB-induced immunosuppression (6–8) and photocarcinogenesis (9–13). As it is shown that UV-induced DNA damage in the form of CPDs plays a critical role in UV-induced immune suppression (15) and carcinogenesis (16–18, 22), the inhibition of UV-induced CPD formation by GTP may be a possible mechanism of prevention of photocarcinogenesis. Moreover, it is important to mention that GTP shows a UV absorption peak at near 270–273 nm, and,

therefore, it is likely that GTP could absorb some wavelengths within the UVB range which are responsible for the DNA damage and erythema formation. It is unlikely that GTP is acting as a sunscreen, because we have earlier shown that GTP protected against a solar-simulated radiation-induced erythema response in humans (23). Additional reasons why GTP possibly may not be acting as a sunscreen include studies where oral consumption of GTP through drinking water to mice was shown to inhibit UV-induced skin tumorigenesis (11–13), immunosuppression (8), and depletion of antioxidant-defense enzymes (24).

In this study where we determined UVB-induced DNA damage, erythema induction was also determined in the same individuals. Treatment with GTP was found to significantly inhibit UVB-induced erythema response in all individuals (Fig. 2) where UVB-induced CPD formation was also found to be inhibited. It is important to mention that with the increase in UV dose, both erythema response and CPD formation in skin increased. GTP treatment was found to inhibit UV-induced CPD formation as well as erythema response. From these data it seems that there may be a direct relationship between CPD formation and erythema response. Because CPDs are instantaneously formed when a DNA molecule absorbs photons, and erythema develops in later stages, it seems that UV-induced pyrimidine dimers mediate erythema development. Thus, our data support a role for human erythema as a good clinical and spectral surrogate for dipyridine DNA photolesions. This view is also supported by recently published studies by Young *et al.* (25). Moreover, animal (*Monodelphis domestica*) studies have also suggested that CPD formation is important in erythema development (26).

On the basis of these observations, it is suggested that the prevention by GTP of UV-induced DNA damage in human skin may prove beneficial for UV-induced nonmelanoma skin cancers and other solar UV-induced skin disorders caused by genetic factors. Moreover, these results also suggest that DNA damage assessed *in vivo* by immunohistochemistry could provide a very sensitive end point for determining the efficacy of protective measures against UVB-induced damage in human skin. Taken together, our study suggests that supplementation with GTPs in skin care products may protect against procarcinogenic DNA photodamage, and that cellular uptake and distribution of polyphenolic compounds is necessary for their optimal photoprotection.

While this article was under review, a publication appeared evaluating in a population-based, case-control study showing how usual tea consumption patterns of an older population ( $n = 450$ ) varied with history of squamous cell carcinoma of the skin (27). This study, though conducted with black tea, showed that tea concentration, brewing time, and beverage temperature have major influences on the potential protective effects of tea in relation to skin squamous cell carcinoma. Thus, detailed investigation on the type of green tea consumption and its preparation technique, especially in regard to its topical application, on human skin squamous cell carcinoma development is warranted.

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