Orally Administered Green Tea Polyphenols Prevent Ultraviolet Radiation-Induced Skin Cancer in Mice through Activation of Cytotoxic T Cells and Inhibition of Angiogenesis in Tumors\textsuperscript{1,2}

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ABSTRACT Green tea polyphenols (GTPs) show promise as anticarcinogenic agents and may prevent the development of solar UV radiation-induced skin cancer. Here we investigated the mechanisms by which GTPs prevent UVB-induced skin cancer in mice. Two groups of 6- to 7-wk-old female SKH-1 hairless mice were UVB irradiated (180 mJ/cm\textsuperscript{2}) 3 times each week for 24 wk. One group consumed water and the other, water containing 2 g/L GTPs. A control group drank water and was not exposed to UVB radiation. UVB-induced tumors and skin biopsies from the control group were analyzed using immunostaining, Western blotting, and gelatinolytic zymography. Oral administration of GTPs reduced UVB-induced tumor incidence (35%), tumor multiplicity (63%), and tumor growth (55%). The GTPs + UVB group had reduced expression of the matrix metalloproteinases (MMP)-2 and MMP-9, which have crucial roles in tumor growth and metastasis, and enhanced expression of tissue inhibitor of MMP in the tumors compared with mice that were treated with UVB alone. The GTPs + UVB group also had reduced expressions of CD31 and vascular endothelial growth factor, which are essential for angiogenesis, and inhibited expression of proliferating cell nuclear antigen in the tumors compared with the UVB group. Additionally, there were more cytotoxic CD8\textsuperscript{T} cells in the tumors of the GTPs + UVB group than in the UVB group and their tumor cells exhibited greater activation of caspase-3, indicating the apoptotic death of the tumor cells. Taken together, these data suggest that in mice, administration of GTPs affects several biomarkers that are involved in UV-carcinogenesis, including inhibition of angiogenic factors and recruitment of cytotoxic T cells in the tumor microenvironment. J. Nutr. 135: 2871–2877, 2005.

KEY WORDS: green tea polyphenols, matrix metalloproteinases, ultraviolet radiation, angiogenesis, cytotoxic T cells

Nonmelanoma skin cancers, including basal and squamous cell carcinoma, represent the most common malignant neoplasms in humans, particularly in Caucasians. Although many environmental and genetic factors contribute to the development of skin cancers, epidemiologic, clinical and biological studies have established that chronic exposure to UV radiation is a well-recognized etiologic agent for both nonmelanoma and melanoma skin cancers, and accounts for ~1.3 million new cases of skin cancers each year in the United States (1). These numbers are probably underestimated because many skin cancers are treated or removed in clinics without being reported to cancer registries. Thus, cutaneous malignancies currently are a major burden on public health and healthcare expenditures. Moreover, it is expected that the dramatic recent increase in the incidence of skin cancer will be sustained due to the aging of the population, the greater amounts of UV radiation reaching the surface of the earth because of depletion of the ozone layer (2–4), and the continuing use of sun tanning devices for cosmetic purposes. The development of effective chemopreventive agents that can reduce or control the risk of UV-induced skin cancer is required to address this pressing public health issue.

In recent years, there has been a great interest in the use of dietary supplements in the form of complementary and alternative medicines that are derived from naturally occurring botanicals for the prevention of UV photodamage, including skin cancer risk. Botanical supplements, specifically dietary botanicals, possessing anti-inflammatory, immunomodulatory, and antioxidant properties are among the most promising group of compounds that can be exploited as ideal chemopreventive agents for skin cancer prevention.

Using in vitro and in vivo model systems, we and others showed that green tea polyphenols (GTPs)\textsuperscript{4} have potent anti-
inflammatory, antioxidant, and immunomodulatory activities (5–7). Topical application of (-)-epigallocatechin-3-gallate (EGCG), a major chemopreventive constituent of GTPs, or oral administration of a crude tea extract of green tea as the sole source of drinking water to SKH-1 hairless mice was shown to inhibit the growth of UV-induced skin tumors (8,9). However, the mechanisms by which the GTPs exert antiphoto- carcinogenic effects in vivo in tumors have not been well defined. We therefore conducted this study using the UVB-induction of skin tumors in SKH-1 hairless mice. Purified GTPs were administered orally in the drinking water (2 g/L) because green tea is commonly consumed as a beverage. Thus, the information generated by this study should be directly relevant to the development of this agent as a complementary and alternative medicine for the prevention of solar UBV-induced cutaneous malignancies.

MATERIALS AND METHODS

Animals. The 6- to 7-wk-old female SKH-1 hairless mice used in this study were obtained from Charles River Laboratories. Mice were housed 5/cage and acclimated for at least 1 wk in the animal research facility before their use in this study. The mice were maintained under standard conditions with a 12-h light/dark cycle, 24 ± 2°C temperature, and 50 ± 10% relative humidity. They consumed an AIN76A control diet and drinking water ad libitum (10). The animal protocol for this study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham, in accordance with the current USDA and Department of Health and Human Service regulations and standards. A group of mice also consumed the AIN76A diet and water ad libitum but without treatment with GTPs and UBV irradiation. At the end of the photocarcinogenesis experiment, mice were killed by exposure to CO2 gas followed by cervical dislocation to confirm their death, as recommended by IACUC.

Experimental design and photocarcinogenesis protocol. After acclimation for at least 1 wk after their arrival in the Animal Research Facility, the mice were divided into 3 age-matched treatment groups for the experiments with 20 mice in each group: control group, control group, and the GTPs+UVB group. GTPs were given in the drinking water as the sole source of drinking water to the mice and continued during the entire photocarcinogenesis protocol. In addition, the mice administered GTPs were switched to GTPs in the drinking water as the sole source of drinking water to the mice and provided in water bottles that were covered with aluminum foil to avoid the possibility of light-induced alterations in the polyphenols, if any. The water was changed at least 3 times/wk. The dose of GTPs (2 g/L) was selected on the basis of our earlier study in which oral administration of GTP was found to prevent UBV induction of markers of tumor promotion (11).

UVB irradiation of mice. Mice were UBV-irradiated as described previously (9). Briefly, dorsal skin was exposed to UV radiation from a band of 4 UBV lamps (Daavlin, UVA/UVB Research Irradiation Unit) from which short wave lengths of UV (<290 nm) not normally present in natural solar light were filtered out using Kodacel cellulose film (Eastman Kodak). The majority of the resulting wave lengths after Kodacel film filtration were in the UBV (290–320 nm) and UVA range with peak emission at 314 nm. The UBV emission was monitored regularly before each UV irradiation with an IL-1700 phototherapy radiometer equipped with an IL SED 240 detector (International Light). All UBV exposures were of 180 mJ/cm².

Evaluation of tumor formation. During the experimental protocol, UBV-irradiated dorsal skin area of the mice was examined for papillomas or tumors on a weekly basis. Growths > 1 mm diameter that persisted for at least 2 wk were defined as tumors and recorded. Tumor data on each mouse were recorded until their yield and size stabilized. At that time point, the dimensions of all of the tumors on each mouse were recorded. Tumor volumes were calculated using the hemispherical model formula: tumor volume = ⅓π(4/3π) (l/2) (w/2) h, where l = length, w = width, and h = height, as described earlier (9). The tumors were then harvested and either snap-frozen in liquid nitrogen or embedded and frozen in OCT medium for further examination.

Gelatinolytic zymography. The activity of MMP-2 and MMP-9 proteins in skin or tumor homogenates was analyzed by gelatinolytic zymography using SDS-PAGE as described previously (12). Briefly, the samples (30–40 μg protein) were resolved on a gel copolymerized with 0.1% gelatin using electrophoresis. After electrophoresis, the gels were washed to remove the SDS. The gels were rinsed twice with zymogen activation buffer and then incubated for 18 h at 37°C in the same buffer. After washing, the gels were stained for 2 h with PfastGel Blue R stain as described by the manufacturer.

Assessment of expression of vascular endothelial cell antigen, CD31. Frozen sections (5 μm thick) were fixed in cold acetone for 10 min. Nonspecific binding sites were blocked in Tris-HCl buffer (pH 7.6) containing goat serum (5%) and bovine serum albumin (0.5%, wt/v). After blocking, the sections were incubated with monoclonal antibodies specific for CD31 in blocking buffer for 1 h. Antibody binding was detected by subsequent incubation of tissue sections with streptavidin-PE-Cy5 secondary antibody for 1 h. The sections were counterstained with Hoechst 33342 which stains nuclei. The intensity of the staining was evaluated using a microscope equipped for immunofluorescence analysis. In addition, vascular permucosal density was assessed by localizing the staining.

Immunohistochemical detection of CD8+ cytolytic T lymphocytes. Immunofluorescent staining was carried out as described for CD31 except that a FITC-conjugated CD38 monoclonal antibody was used.

Preparation of skin and tumor lysates for Western blotting. The tumors and epidermal skin from the control group were used for lysate preparation as described previously (13,14). The epidermis was separated from the whole skin as described earlier (15). Tissue samples were pooled from 6 mice in each experimental group and lysates were
from gel to membrane, the blots were stripped and reprobed for then visualized using the ECL detection system (Amersham Life blot was then washed with PBS and incubated with secondary anti-
body conjugated with horseradish peroxidase. Protein bands were then visualized using the ECL detection system (Amersham Life Science). To verify equal protein loading and transfer of proteins from gel to membrane, the blots were stripped and reprobed for β-actin using an anti-actin rabbit polyclonal antibody.

Statistical analysis. Tumor incidence in the UVB and GTPs+UVB groups was compared by χ² analysis. Tumor multiplicity data were analyzed using the Wilcoxon rank-sum test, and tumor volume was analyzed using ANOVA followed by Tukey’s test. Statistical analysis of tumor data was performed at the termination of the experiment. A simple ANOVA followed by Tukey’s test was also used to calculate the significance of the data obtained from Western blot analysis. To compare the intensity of different biomarkers in immunostaining, at least 4 different areas from each section were selected and the color intensity of positive cells was measured and quantified using the OPTIMAS 6.2 computerized software program. Differences with P < 0.05 were considered significant.

RESULTS

GTPs prevent UVB-induced skin carcinogenesis. The administration of GTPs in drinking water reduced UVB-induced skin carcinogenesis in terms of tumor incidence (Fig. 1, Panel A), tumor multiplicity (Fig. 1B), and tumor size (Fig. 1C) compared with mice not administered GTPs. Oral administration of GTPs resulted in a 35% (P < 0.05) lower tumor incidence at the termination of the experiment at 24 wk (Fig. 1A). The mice that did not receive GTPs had a 100% tumor incidence during wk 16 of tumor promotion, whereas the mice administered GTPs did not have 100% tumor incidence until the end of wk 24 (P < 0.05) when the tumor yield was stabilized. Thus oral administration of GTPs inhibited UVB-induced tumor multiplicity (63%, P < 0.005) compared with the group exposed to UVB but not administered GTP (Table 1). Compared with the group of mice not administered GTPs, the size of the tumors in the mice receiving GTPs was reduced when determined in terms of total tumor volume/group (Table 1, 62%, P < 0.005), tumor volume/tumor-bearing mouse (Fig. 1C, 55%, P < 0.005) and mean tumor volume/tumor (Table 1, 38%, P < 0.05). The mice in the control group that were not irradiated did not develop tumors within the 24-wk study period. During the photocarcinogenesis protocol, body weights and food and fluid intakes did not differ among the 3 groups (data not shown).

GTPs inhibit MMP-2 and MMP-9 expression while increasing TIMP1 in tumors. Immunohistochemical staining revealed the presence of MMP-2 positive cells in the tumors of mice administered UVB or GTPs+UVB, but they were rarely observed in the control group (Fig. 2, Panel A). However, there were fewer MMP-2-positive cells in the tumors of the mice administered GTPs+UVB (P < 0.01) and their distribution was more sporadic (Fig. 2A) compared with the UVB group. Western blot analysis indicated a higher expression of MMP-2 in UV-induced tumors compared with the epidermal skin of the control group (Fig. 3, Panel A, P < 0.005) and confirmed the inhibition (P < 0.05) of MMP-2 expression in the tumors of mice that were administered GTPs+UVB. Analysis of the activity of the MMP-2 protein in the homog-enates by gelatinolytic zymography (Fig. 3B) clearly indicated that the activity was markedly inhibited in the mice admin-
istered GTPs+UVB compared with UVB alone. Similarly, the administration of GTPs inhibited the activity of MMP-9 (gelatinase B) in the tumors (Fig. 3D; P < 0.001), and also inhibited MMP-9 protein expression as determined by immunostaining (Fig. 2B; P < 0.01) and Western blot analysis (Fig. 3C, P < 0.005) compared with the UVB group. The immu-

nohistochemical staining intensity of MMP-9 in the tumors obtained from the mice of UVB group was higher (P < 0.001) than that in control skin; however, the MMP-9 staining in the tumors obtained from the mice administered GTPs+UVB was widely scattered and fainter than the staining pattern of the tumors obtained from the UVB group. Further, administration of GTPs (GTPs+UVB group) resulted in an ~200% increase in the activity of MMP-9 in the tumors obtained from the mice administered GTPs+UVB.
in TIMP1, the naturally occurring inhibitor of these proteins (Fig. 3E, P < 0.005) compared with UVB group; this may have contributed to the inhibition of the expression of both MMP-2 and MMP-9 in these mice. Thus, our data suggest that one mechanism by which the administration of the GTPs acts to inhibit UVB-induced tumor formation is through inhibition of the synthesis or secretion of MMP-2 and MMP-9 proteins, thus curtailing the proangiogenic stimuli that promote tumor growth (16–19).

**GTPs inhibit the expression of CD31 and VEGF in tumors.** Immunohistochemical analysis of the expression of CD31 indicated intense staining of the tumor cells in the mice of the UVB group (Fig. 4, Panel A) which was greater than that of control skin (P < 0.001). Staining of CD31 was weak in the tumors of the mice administered GTPs+UVB (Fig. 4 A). Moreover, the staining pattern of CD31 in the tumor tissue sections from mice of the UVB group revealed elongated vessels that were often arranged in clusters (Fig. 4A). In contrast, the CD31 staining pattern of the tumors in the mice administered GTPs indicated inhibition of the peritumoral vascular density and a reduction in the clusters and elongated structures of vessels in tumors compared with the UVB group. This suggests inhibition of the formation of new vasculature and thus inhibition of tumor growth by GTPs.

The evidence supporting a central role for VEGF in tumor-induced angiogenesis, tumor growth, and metastasis thus identifies VEGF as a promising target for antitumor therapy (20,21). Western blot analysis revealed that UVB-induced tumors overexpressed VEGF compared with normal skin biopsies and that oral administration of GTPs inhibited the expression of VEGF in the UVB-induced tumors (Fig. 5A, P < 0.005).

**GTPs inhibit PCNA expression in tumors.** The expression of PCNA is an indicator of the proliferative activity of tumor cells. Western blot analysis indicated that the levels of PCNA were higher in the UVB-induced tumors than in the skin biopsies of control mice (Fig. 5B). Administration of GTPs inhibited the enhancement of the expression of PCNA in the UVB-induced tumors (P < 0.005).

**GTPs increase the numbers of cytotoxic T lymphocytes in skin tumors.** Because cytotoxic T cells (CD8 T cells) are tumoricidal in nature, we determined whether administration of GTPs induces the recruitment of cytotoxic T lymphocytes to the tumor site using immunofluorescence analysis. The intensity of fluorescence staining and the number of CD8 T cells were higher in tumors treated with GTPs+UVB compared with the UVB group of mice (Fig. 4B). These results indicate another major pathway by which GTPs can inhibit tumor growth.

**GTPs induce the activation of caspase-3 in tumors.** Because we observed that treatment with GTPs increased the numbers of CD8 T cells in the tumor microenvironment, and these cells are cytotoxic to tumor cells, it is possible that the tumor cells are undergoing apoptosis. Activation of caspase-3 is a terminal event in apoptotic cells. We found higher levels of activation of caspase-3, as indicated by its cleavage in the UVB-induced tumors of mice administered GTPs (Fig. 5C) than in the mice with UVB-induced tumors or mice not given GTPs. These data suggest that induction of cell death or apoptosis mediated by the activation of caspase-3 in tumor cells contributes to the inhibition of UVB-induced tumor growth with administration of GTPs. The activation of caspase-3 was not evident in the skin of the control group.

**DISCUSSION**

In our continuing efforts to develop effective dietary botanical supplements as chemopreventive agents for the pre-

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**TABLE 1**

*Photoprotective effect of GTPs on UVB-induced skin tumors in mice at the end of the photocarcinogenesis protocol*\(^1,2\)

<table>
<thead>
<tr>
<th></th>
<th>UVB alone</th>
<th>GTPs + UVB</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-bearing mice/group, n</td>
<td>20</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>Total tumors/group, n</td>
<td>362</td>
<td>132*</td>
<td>63</td>
</tr>
<tr>
<td>Total tumor volume/group, mm(^3)</td>
<td>9230</td>
<td>3507*</td>
<td>62</td>
</tr>
<tr>
<td>Mean tumor volume/tumor, mm(^3)</td>
<td>26 ± 7</td>
<td>16 ± 4**</td>
<td>38 ± 4</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SEM or n. Asterisks indicate different from UVB alone: * P < 0.005, ** P < 0.05.

\(^2\) Number of tumors and tumor volume were recorded at 24 wk when tumor yield and growth had stabilized.
vention of photocarcinogenesis, we assessed the efficacy of oral administration of GTPs. Using a mouse model, we found that oral administration of GTPs resulted in significant protection against photocarcinogenesis in terms of tumor incidence, tumor multiplicity, and tumor growth (Fig. 1, Table 1). This degree of prevention was less than that reported for topical application of EGCG (9). Presumably, this difference is associated with the higher concentration of EGCG available in the skin after topical application compared with oral administration. The chemopreventive effects we observed were appreciable, however, and directly relevant because green tea is commonly consumed as a beverage. Thus, administration of GTPs as a beverage may serve as a complementary and alternative medicine for the prevention of skin cancer.

In an effort to define the pathways by which oral administration of GTPs inhibits UV-induced skin carcinogenesis, we examined biomarkers of angiogenesis, cellular proliferation, and tumor cell death or apoptosis. The protein expression and secretion of both MMP-2 and MMP-9 were markedly lower in tumors from mice administered GTPs, and the activity of these gelatinases also was reduced. The inhibition of MMPs was accompanied by elevated expression of their natural inhibitor, TIMP1. This suggests one mechanism by which GTPs inhibit UV-induced tumor growth. These data are in concordance with our previous study of in vitro treatment of human prostate carcinoma cells (DU145) with (-)-epigallocatechin-3-gallate (a major constituent of GTPs) in which we also observed a downregulation of MMP-2 and MMP-9 protein expression as well as downregulation of their activities (12). Furthermore, our in vivo tumor model revealed that administration of GTPs in the drinking water inhibited the expression of vascular endothelial cell antigens, such as CD31 and VEGF, in the UVB-induced tumors. These proteins play an important role in tumor growth, invasion, and metastasis due to their promotion of the new vasculature formation that supports the growth of the tumor. Thus, administration of GTPs in the drinking water has a strong antiangiogenic effect and has the potential to check the growth, or cause regression, of the
tumors through this mechanism. Whether the GTPs affect the expression of each of these proteins separately or target a key molecule that regulates angiogenesis is yet to be resolved.

The inhibition of PCNA in the microenvironment of the tumors supports the concept that the proliferation of the tumor cells is suppressed. Again, this may reflect the action of the GTPs on other mechanisms, such as angiogenesis or may be a direct effect of the inhibitory action of GTPs. Such an effect on the expression of PCNA would have the potential to inhibit both UVB-induced skin carcinogenesis and the growth of the tumors once initiated.

CD8+ T cells are the effector cells in the cytotoxic response of the host to UV-induced skin tumor cells; they play an important role in protection against tumor immunity at least for skin tumors induced by chronic UV exposure (22). The ability of oral administration of GTPs to enhance the infiltration or recruitment of higher numbers of CD8+ T cells in the tumor microenvironment may act to enhance the immunosurveillance that is mediated by these cells, thereby reducing the incidence of tumors. It also has the potential to inhibit UV-induced tumor growth and promote regression of developing tumors. Finally, our studies established that the level of activation of caspase 3 was enhanced in tumors from mice admin-

thesized GTPs. This indicates that the cells are undergoing apoptosis (23,24) and could reflect apoptosis induced by the activity of the cytotoxic T cells and/or a direct effect of the GTPs. Similar studies were conducted with EGCG (a major component of GTPs) in which the chemopreventive effect of topical application of EGCG in a hydrophilic cream was evaluated with an identical photocarcinogenesis protocol and biomarkers of angiogenesis and cytotoxic T cells in tumors (25). The degree of chemopreventive effect of GTPs was lower than that with topical application of EGCG. Presumably, this difference may be due to the higher concentration of EGCG available in the skin with topical application compared with oral administration of GTPs. However, the chemopreventive effect of orally administered GTPs in mice was notable against the biomarkers studied in this study; it can be considered as important as EGCG because GTPs are affordable, less costly than pure EGCG, and can be easily obtained from green tea beverage for the prevention of UV-induced skin cancer and other harmful effects of UV radiation. On the basis of the information obtained in this study, it is suggested that the daily consumption of 5–6 cups (1 cup = 150 mL) of green tea (1 g green tea leaves/150 mL) by humans may provide the same level of GTPs in the in vivo human system and should provide similar protection against harmful effects of UV radiation. However, the magnitude of chemopreventive effect of GTPs may differ from person to person due to differences in race, and UV radiation intensity and exposure time.

In summary, our data suggest that GTPs can act to prevent both UVB-induced skin carcinogenesis and the growth of the tumors once established. They further indicate that GTPs can act through at least 2 mechanisms in our in vivo tumor model system: 1) inhibition of the proangiogenic stimuli, and 2) promotion of cell death or apoptosis of the tumor cells. Thus, green tea polyphenols have potential for development as a complementary and alternative medicine to prevent UV-induced nonmelanoma and melanoma skin cancers.

**LITERATURE CITED**


