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Pharmacokinetics of the green tea derivative, EGCG, by the topical route of administration in mouse and human skin

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Abstract *Purpose*: (−)-Epigallocatechin gallate (EGCG), the main physiologically active polyphenol of green tea, is associated with antitumor and antimutagenic activities. The goal of this study was to determine the stability and pharmacokinetic parameters of pure EGCG administered topically to human and mouse skin. *Methods*: EGCG was investigated by measuring drug levels of a 10% ointment formulation stored under different conditions over a period of 6 months. To determine pharmacokinetic parameters of EGCG following topical application, EGCG was applied as 10% EGCG in hydrophilic ointment USP to full-thickness mouse or human skin in vitro. The transdermal and intradermal. Penetration of EGCG was measured by reverse phase HPLC assays at different time-points. *Results*: The stability of EGCG in hydrophilic ointment USP was dependent on time, temperature and the degree of oxidation. For example, 10% EGCG was lost after 2 days at 37 °C, but the same formulation supplemented with 0.1% butylated hydroxytoluene (BHT) had significantly longer stability with ≥90% EGCG remaining after 130 days at 37 °C. Topical application of EGCG in hydrophilic ointment USP to human or mouse skin resulted in substantial intradermal uptake of up to 1–20% of the applied dose. However, transdermal penetration was observed only in mouse skin. *Conclusion*: The present study showed that topical application of EGCG in hydrophilic ointment USP achieved high concentrations in skin but negligible systemic availability. The drug was susceptible to oxidation, but if supplemented with BHT, the hydrophilic ointment formulation could potentially be used in clinical trials of skin cancer prevention.

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Key words Green tea · Catechins · Skin · Topical · Chemoprevention

Introduction

Tea (*Camellia sinensis*) is one of the most widely consumed beverages in the world. Epidemiological studies have detected an association between tea consumption and decreased cancer risk [20]. Furthermore, green tea polyphenols have been reported to have antimutagenic and antitumor activities [1, 3, 10, 13, 16]. Recent studies have shown an antitumor effect of green tea polyphenols in mouse models of skin carcinogenesis [9]. Topical application of green tea polyphenolic extracts to mouse skin inhibits tumorigenicity of polycyclic aromatic hydrocarbons and tumor promotion by 12-O-tetradecanoylphorbol-13-acetate [21]. (−)-Epigallocatechin gallate (EGCG, Fig. 1) is the main polyphenolic fraction of green tea. EGCG inhibited tumor promotion induced by teleocidine in a two-stage experiment using the mouse skin chemical carcinogenesis model. Pure EGCG in acetone applied topically to mouse skin also prevents UVB-induced photocarcinogenesis without severe side effects, whereas oral dosing is inactive [2].

These recent findings suggest that EGCG may have the potential to play an important role in skin cancer treatment and prevention. However, there is no information on the absorption and permeability parameters of EGCG in mouse or human skin. Here, we describe a novel HPLC method for the detection of EGCG in human and mouse skin. This method was applied to pharmacokinetic studies of EGCG, administered topically to mouse and human skin as a 10% EGCG in hydrophilic ointment USP, or as a solution in acetone. In addition, the stability of 10% EGCG ointment under different storage conditions was investigated. An understanding of these parameters will be important in determining the potential chemoprotective actions of this compound.

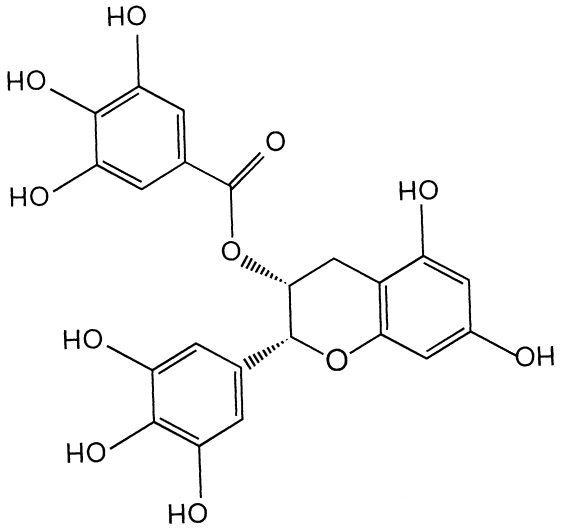

Fig. 1 The structure of EGCG

Materials and methods

Chemicals

EGCG was purified from a green tea blend provided by the Royal Estates Tea Company, Division of Thomas J. Lipton (Englewood Cliffs, NJ). The procedure involved repeated extraction followed by column chromatographic purification, and is described elsewhere [2, 16]. The structure and purity (>99%) of EGCG were confirmed by MS and MPLC analyses, respectively (data not shown). EGCG (10% w/w) in hydrophilic ointment USP (E. Fougera & Company, Melville, NY) was formulated for topical application with or without butylated hydroxytoluene (BHT, 0.1 or 0.5% w/w; Sigma Chemical) as a preservative. The topical dose was delivered in 200 µl of 10% (w/w) EGCG ointment formulation. This dose contained 17 mg pure EGCG. For the HPLC assays, an EGCG storage solution containing ascorbic acid (20% w/v) and EDTA (0.1% w/v) was prepared in sodium phosphate buffer (0.4 *M*, pH 3.6). All other reagents were of the highest purity available.

In vitro skin penetration studies

Female BALB/c mice weighing 20–25 g were purchased from Harlan Sprague Dawley (Indianapolis, Ind.). Human skin from a Caucasian cadaver donor was kindly supplied by Dr. Richard Sobonya, Department of Pathology, College of Medicine, The University of Arizona, Tucson, Ariz. The specimens were stored overnight for 12–14 h at 4 °C in a transport medium containing phosphate-buffered saline (PBS), pH 7.4, supplemented with l ml of an antibiotic mixture containing penicillin 1000 units/ml and streptomycin 10 000 µg/ml per 25 ml of medium. Prior studies had shown no loss of cell viability or change in marker drug (dexamethasone) transdermal penetration for these stored human skin samples compared with those prepared and tested immediately after harvest. Excessive fat and connective tissue were removed from the human skin using a scalpel. Full-thickness mouse skin was removed from BALB/c mice dehaired 24 h prior to the experiment.

Transdermal permeability and absorption of EGCG were studied using the Skin Penetration System 3 (Laboratory Glass Apparatus, Berkeley, Calif.). Briefly, sterile PBS (3.5 ml) was placed in the lower reservoir compartment along with a magnetic stirring bar. A full-thickness mouse or human skin sample was sandwiched between the lower penetration cell reservoir and the glass cell top containing the drug sample, dermal side down. The top glass sample reservoir was secured in place with a clamp. EGCG was then applied using a positive displacement pipette (Gilson Model M-250, Woburn, Mass.) to the epidermal side of the exposed skin in acetone (10 mg or 50 mg in 200 µl) or in the hydrophilic ointment USP (10% w/w, 200 µl, 0.05% w/w BHT). The cell top was covered with Parafilm and the system was incubated at 37 °C with constant stirring. Following in vitro incubation, skin samples were rinsed with PBS, weighed and stored at −80 °C until analysis. The receptor fluid was mixed with ascorbic acid/EDTA solution (20 µl/ml) and stored at −80 °C until analysis.

HPLC

HPLC analyses were performed on a Perkin-Elmer Biocompatible 250 Binary Pump (Norwalk, Ct.), Hitachi autosampler (Model AS-2000, Danbury, Ct.) and a Hewlett-Packard 1050 UV-VIS detector (Avondale, Pa.) at a fixed wavelength of 280 nm. The system was controlled with PE Nelson TurboChrom 4 software. The column used was an Absorbosphere HS[7] C18 (Alltech Associates, Deerfield, ll.), 150 mm × 4.6 mm, 5 µm particle size. The mobile phase consisted of 82% H_3PO_4 (0.05%) and 18% acetonitrile at a flow rate 1.4 ml/min.

Standard solutions of EGCG (1 mg/ml) were prepared daily and contained ascorbic acid/EDTA solution (20 µl/ml) to prevent oxidation. Calibration curves were prepared daily by spiking appropriate amounts of EGCG to (1) PBS, (2) pulverized skin or (3) 10 µl hydrophilic ointment USP. Blank extracts of skin and hydrophilic ointment, USP were also prepared and analyzed concurrently.

Sample preparation

Skin samples (~40 mg) were pulverized in liquid nitrogen. Ascorbic acid/EDTA solution (100 µl, 2%) was added and the samples were twice extracted with 800 µl ethyl acetate as described above. EGCG that penetrated through the skin was measured by direct injection of the receptor fluid (PBS) onto the HPLC column.

To determine the stability of EGCG in the ointment formulation, samples of EGCG in hydrophilic ointment USP (10% w/w) were analyzed by HPLC over a period of 6 months at three different storage temperatures: 4 °C, 25 °C and 37 °C. Briefly, ointment (10 µl) was vortexed with 100 µl ascorbic acid/EDTA solution (2% v/v) and extracted twice with ethyl acetate as described above. A 10-µl aliq uot of the resulting sample was injected onto the HPLC column and the peaks were analyzed and quantified as before.

Results

HPLC

A typical chromatogram from a mouse skin extract is shown in Fig. 2. The final chromatographic and extraction conditions were modified from those of Zhu et al. [22] and Lee et al. [7] to improve peak shape and the separation of EGCG from endogenous substances in plasma or skin. The overall chromatographic time was 10 min with EGCG eluting as a symmetrical peak at a retention time of 5 min. The extraction efficiency of EGCG from skin was 83 ± 8% ($n = 3$). Control experiments showed that no detectable EGCG could be recovered from skin treated with 10% EGCG in hydrophilic ointment but then immediately rinsed three times with PBS. The best separation of EGCG was achieved on the C18 column eluted with a mobile phase comprising acetonitrile and 0.05% H_3PO_4 (18:82 v/v).

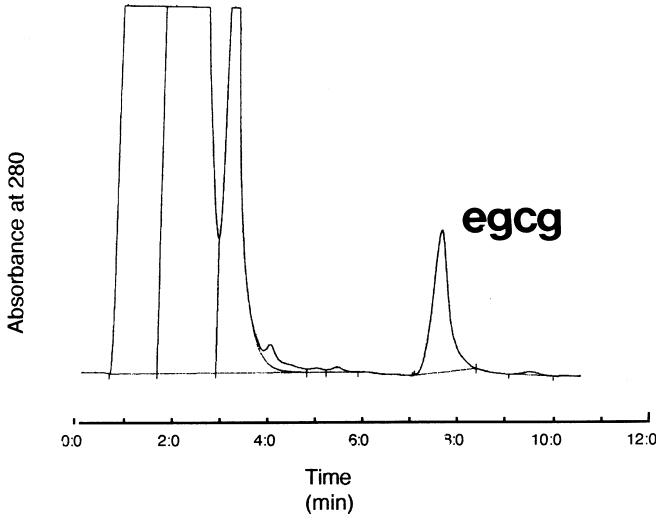

Fig. 2 Chromatogram of human skin extract following EGCG topical administration in vitro

Extracts from control skin samples showed no interfering peaks at the retention time of EGCG. The detection limit of this assay for EGCG in skin samples was 100 ng/mg of skin, and for the receptor fluid, 10 ng/ml.

In vitro skin penetration studies

In vitro penetration of EGCG dissolved in acetone (10 mg, 50 mg) or of 10% EGCG ointment (200 μl) through full-thickness mouse skin was measured by HPLC. Figure 3A shows the penetration profiles for three different formulations of EGCG in the mouse skin. As expected, the absorption of EGCG in acetone into the mouse skin was rapid and reached plateau intradermal concentrations of approximately 0.5 mg/cm^2 and 0.9 mg/cm^2 of mouse skin for doses of 10 mg/cm^2 and 50 mg/cm^2, respectively. This correlated with an intradermal uptake of 5% of the total dose applied for the 10 mg application, and 1.8% for the 50 mg application of EGCG. The rate of intradermal absorption of EGCG, applied as the 10% ointment, was slower but more complete. The maximal concentration of EGCG achieved in mouse skin from the ointment formulation was 3.3 mg/cm^2. This was reached after 24 h of incubation and represents an intradermal uptake of 19% of the total topical dose applied to the skin.

Similar topical absorption studies were also performed with human cadaver skin. Rapid absorption of EGCG dissolved in acetone was observed in human skin (Fig. 3B). There was a much slower absorption of EGCG following topical application of the ointment-based vehicle. The plateau intradermal concentration of EGCG applied as the 10% EGCG ointment to human skin was 0.15 mg/cm^2, representing 0.9% of the dose applied after 24 h of incubation.

In vitro transcutaneous permeability of EGCG through mouse skin was also measured by HPLC.

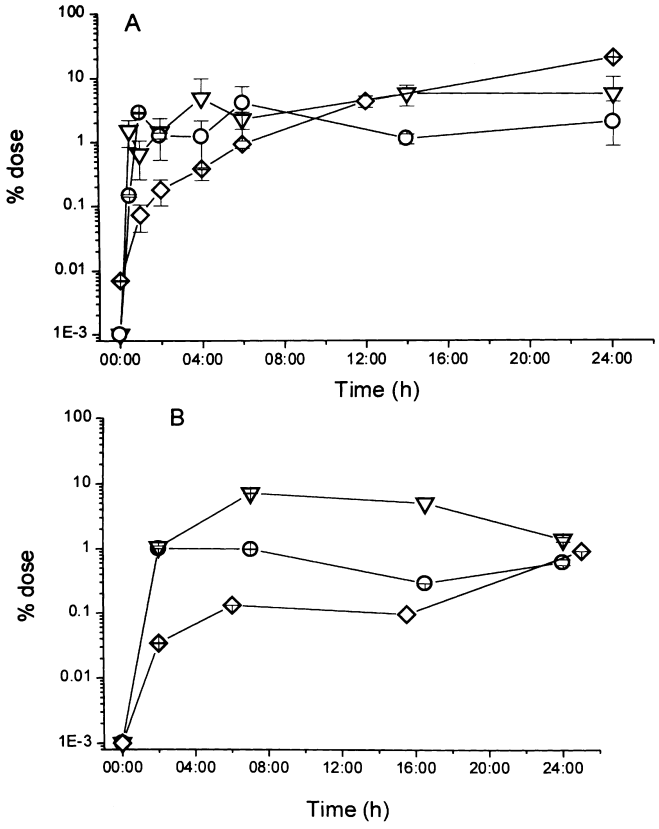

Fig. 3A,B Absorption of EGCG after topical application of EGCG in acetone at concentrations of 10 mg/cm^2 (∇) and 50 mg/cm^2 (○), or 17 mg/cm^2 from the 10% EGCG hydrophilic ointment (◊), into mouse skin (**A**) and human skin (**B**). Each value represents the mean of four determinations from two different experiments

Figure 4 shows the time-course of EGCG skin penetration from three different concentrations. The same formulations of EGCG were tested as in previous experiments. Low concentrations of 0.14 μg/ml, representing 0.01% of the topical dose of EGCG, were

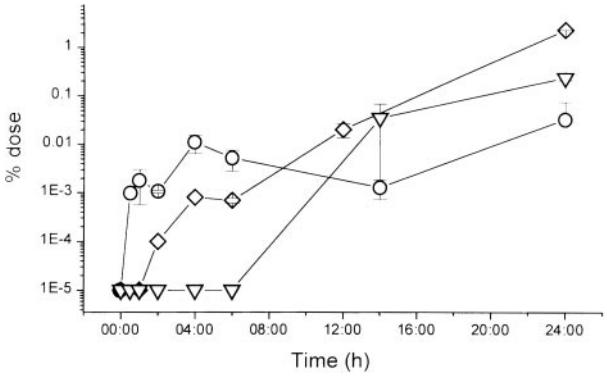

Fig. 4 Transdermal penetration of EGCG after topical application in EGCG in acetone at concentrations of 10 mg/cm^2 (∇) and 50 mg/cm^2 (○), or 17 mg/cm^2 from the 10% EGCG hydrophilic ointment (◊) using full-thickness mouse skin. Each value represents the mean of four determinations from two different experiments

recovered in the transdermal receptor fluid after 4 h of incubation. When EGCG was applied in the ointment formulation, a maximum uptake of 385 μg, representing 2.26% of the applied dose, was reached after 24 h of incubation. No transdermal penetration was observed after 6 h of incubation following the application of 10 mg EGCG dissolved in acetone, and less than 1% of the total dose was detected in the receptor fluid after 24 h of exposure. However, penetration through mouse skin was faster, especially when the higher dose of 50 mg EGCG in acetone was applied. Following a 30-min incubation, low levels of 0.5 μg/ml or 0.01% of the dose applied were recovered from the incubation fluid, and a maximum of 16.8 μg/ml or 0.03% of the applied dose had penetrated through the skin during 24-h of incubation.

The transcutaneous permeability of 10% EGCG in the ointment formulation was also measured in human cadaver skin. In four experiments, no EGCG was detected in the incubation fluid following a 24-h exposure. Overall, these results show that EGCG uptake into and through mammalian skin is dependent on both the vehicle used and the length of topical exposure.

Stability of 10% EGCG ointment

The stability of 10% EGCG in hydrophilic ointment USP (w/w) was evaluated over 6 months. The ointment was protected from light and stored at different temperatures (−20 °C, 4 °C, 25 °C, 37 °C) to determine the effect on the stability of EGCG in the cream. Extraction and HPLC assays to determine levels of EGCG in cream were performed once every 2 weeks for up to 6 months. After 2 weeks, ointment samples stored at 37 °C and 25 °C changed color from white to light brown and reduced concentrations of EGCG were recovered from these samples. Only 2 days were required for a 10% loss of EGCG under these conditions. The same trend for temperature-dependent stability was observed in other ointment samples stored under different conditions after 4–6 weeks (Table 1). The ointment samples stored at −20 °C changed consistency, becoming gritty, while the samples kept at 4 °C did not change in color or texture.

Because oxidation of EGCG was suspected at the higher temperatures, we decided to prepare new formulations of EGCG in hydrophilic ointment USP (10%, w/w) supplemented with BHT (0.05% or 0.1%, w/w) to prevent oxidation of EGCG. The ointments supplemented with BHT and stored at 4 °C were highly stable and exhibited no change in color or consistency for up to 6 months (Table 1). In this case, stability was dependent on the concentration of BHT. The loss of EGCG in the samples supplemented with 0.05% BHT and stored at 37 °C was 10% after 28 days, and about 38% at 5 months. Even greater stability was observed for EGCG creams containing 0.10% BHT (w/w) with a 10% loss observed only after 130 days of storage at 37 °C (Table 1).

Table 1 Stability of EGCG in hydrophilic ointment USP (10%, w/w) without or with BHT (0.05%, 0.10%, w/w) under different storage temperatures

Temperature (°C)	Time (days) for 90% EGCG remaining		
	0% BHT	0.05% BHT	0.10% BHT
−20	> 180	> 180	> 180
4	101	> 180	> 180
25	7	100	> 180
37	2	28	130

Discussion

Tea consumption has been inversely correlated with carcinogenesis in several epidemiologic studies [20]. Laboratory studies have demonstrated inhibition of tumorigenesis in animal models, especially for mouse skin tumors induced by topical treatment with a variety of polycyclic aromatic hydrocarbons [4–6, 17–19]. In these studies, green tea polyphenols reduced skin carcinogenesis when administered orally [17] or topically [4, 21]. Our group also has demonstrated prevention of UVB-induced photocarcinogenesis in BALB/c mice using topical EGCG dissolved in acetone [2]. However, oral administration of EGCG in the drinking water, at doses up to 2.8 mg/day, did not block UVB-induced skin tumor formation in the same study [2]. The lack of oral efficacy for EGCG may be due to the low (2%) oral bioavailability of green tea polyphenols in vivo [7, 11, 15]. Direct antitumor effects also have been observed for six different green tea catechins tested in four human tumor cell lines in vitro, with EGCG being the most potent [16]. In addition, EGCG is active as a modulator of multidrug resistance in doxorubicin-resistant murine sarcoma and human colon cancer cell lines [12]. These findings suggest that green tea polyphenols and especially EGCG have activity in cancer prevention models, and in the treatment of malignant cells in both animal and in vitro models.

Despite these biological data, relatively little is known about the pharmacology and pharmacokinetics of EGCG in any of these model systems. In rats, Matsumoto et al. have reported 20% elimination of EGCG 2 h after oral administration [8]. However, the actual oral bioavailability of EGCG was not reported. Subsequently, Unno and Takeo showed that EGCG is absorbed, reaching a peak plasma level 1 h after administration of a 50-mg oral dose dissolved in 2 ml of water [14]. Using an HPLC method, a peak plasma level of about 200 ng/ml was produced by this dose, but since the pure drug was not parenterally administered, the true oral bioavailability could not be determined. A human study of plasma and urinary tea polyphenol levels in volunteers given green tea orally has similarly shown that EGCG is detectable in the plasma, but distribution into the skin was not measured [7].

The current findings expand this database considerably. The topical pharmacokinetic data show that the drug is efficiently absorbed into and through intact mouse skin. Intradermal drug absorption in mouse skin was considerably improved with an ointment formulation compared to acetone applications. In contrast, transdermal penetration through full-thickness human cadaver skin was less efficient for both formulations, even though intradermal uptake was only slightly lower than that seen in mice. This suggests that human skin provides a much more efficient barrier than mouse skin for preventing systemic availability from topically applied EGCG.

Another important physical observation is the temperature- and oxidation-dependent stability of EGCG when admixed into a standard topical ointment vehicle. Thus, without the addition of the antioxidant BHT, a 10% loss of EGCG occurred in only 2 days at 37 °C. This decay was greatly retarded at lower temperatures and with the addition of BHT (0.05% or 0.10%, w/w). With the latter BHT concentration, and with storage at 25 °C, >90% of the original EGCG concentration was present 6 months after preparation. This suggests that topical EGCG formulations in hydrophilic ointment USP could be used in clinical trials if precautions are taken to avoid heat and oxidation.

In summary, the current results suggest that the green tea-derived polyphenol EGCG is poorly absorbed systemically following topical application to human skin. However, the ointment formulation facilitates substantial intradermal uptake into mouse and human skin. A 10% topical formulation of EGCG (w/w) in hydrophilic ointment USP has short stability unless stored at low temperature and in the presence of an antioxidant such as BHT. These results suggest that properly formulated topical EGCG formulations, stored at low temperature, could be used for studies investigating skin cancer prevention strategies with green tea polyphenolic compounds.

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