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Prevention of UVB-induced immunosuppression in mice by the green tea polyphenol (–)-epigallocatechin-3-gallate may be associated with alterations in IL-10 and IL-12 production

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UV exposure of the skin, particularly UVB (290–320 nm), causes adverse biological effects, including alterations in cutaneous immune cells, photoaging and photocarcinogenesis. Several studies have shown that polyphenolic compounds isolated from green tea afford protection against UVB-induced inflammatory responses and photocarcinogenesis in murine models. In this study we show that topical application of (–)-epigallocatechin-3-gallate (EGCG) (3 mg/mouse), a major polyphenolic component of green tea, before a single low dose UVB exposure (72 mJ/cm^2) to C3H/HeN mice prevented UVB-induced inhibition of the contact hypersensitivity response and tolerance induction to the contact sensitizer 2,4-dinitrofluorobenzene. Topical application of EGCG before UVB exposure reduced the number of CD11b+ monocytes/macrophages and neutrophils infiltrating into skin inflammatory lesions, which are considered to be responsible for creating the UV-induced immunosuppressive state. In addition, application of EGCG before UVB exposure decreased UVB-induced production of the immunomodulatory cytokine interleukin (IL)-10 in skin as well as in draining lymph nodes (DLN), whereas production of IL-12, which is considered to be a mediator and adjuvant for induction of contact sensitivity, was found to be markedly increased in DLN when compared with UVB alone-exposed mice. Taken together, our data demonstrate that EGCG protects against UVB-induced immunosuppression and tolerance induction by: (i) blocking UVB-induced infiltration of CD11b+ cells into the skin; (ii) reducing IL-10 production in skin as well as in DLN; (iii) markedly increasing IL-12 production in DLN. Protection against UVB-induced immunosuppression by EGCG may be associated with protection against UVB-induced photocarcinogenesis.

Introduction

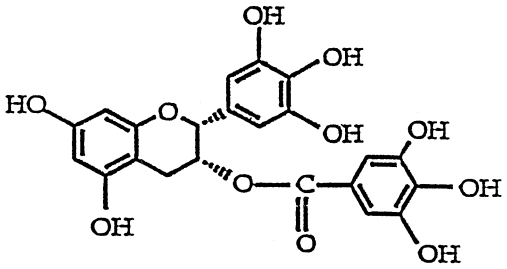
UV radiation, particularly UVB (290–320 nm) within the solar spectrum, which has suppressive effects on the immune system (1), can act as a tumor initiator (2), a tumor promoter (3) and a co-carcinogen (4,5). These UV-induced alterations play an important role in the generation and maintenance of UV-induced neoplasms (6). Studies with biopsy-proven skin cancer patients have indicated that UV-induced immunosuppression is a risk factor for skin cancer development in humans (7). UV irradiation can induce immunosuppression to a contact sensitizer through different mechanisms that operate primarily by influencing the type or function of the cells presenting the antigen stimulus. Single low dose UV exposure of the skin drastically reduces the number of epidermal Langerhans cells and application of haptens to the cutaneous areas depleted of Langerhans cells by UV results in an absence of contact hypersensitivity (CHS) (8). UV irradiation drastically reduces the number of Langerhans cells (8–10) either by induced migration (11,12) or by cell death (13).

Exposure of murine skin to low doses of UVB radiation before sensitization with hapten reduces the ability of antigen-presenting cells (APC) in the draining lymph nodes (DLN) to initiate CHS responses *in vivo* and results in the induction of hapten-specific suppressor T cells (14). DLN cells produce less Th1- and Th2-associated cytokines in response to APC from UV-irradiated mice compared with APC from unirradiated, fluorescein-sensitized mice (15). Because UVB is almost completely absorbed within the epidermis and cannot directly affect cells outside the irradiated layer, one potential mechanism for UV-induced inhibition of CHS involves the release of immunomodulatory cytokines by epidermal cells (16). The primary role of the cytokine interleukin (IL)-10, produced by keratinocytes, has been suggested to be to mediate UV-induced systemic immunosuppression (17). Intraperitoneal injection of IL-10 into mice has been shown to suppress the effector phase, but not the induction phase, of CHS and also the induction phase of delayed type hypersensitivity (18). However, it has also been suggested that the systemic suppression of CHS is not mediated by IL-10 (19). Further, i.p. injection of neutralizing anti-IL-10 antibody prevents UV-induced tolerance in mice (20).

IL-12 protein is composed of two disulfide bonded chains, p40 and p35 (21,22), thus making it heterodimeric in nature. Only the 70 kDa dimer shows biological activity, whereas neither chain alone is bioreactive, although a possible inhibitory function of the IL-12 p40 chain homodimer has been identified (23). An important role of IL-12 has been demonstrated in the induction and elicitation of the contact sensitization reaction (24). It has been shown that IL-12 is produced in regional lymphatic organs and the spleen following the application of allergen (24). Injection of anti-IL-12 mAb before allergen painting prevents sensitization *in vivo*, whereas administration of IL-12 breaks UV-induced tolerance in mice, thus demonstrating the important adjuvant function of this cytokine (24,25). Intraperitoneal injection of murine rIL-12 prevents UV-induced local immunosuppression and overcomes UV-induced hapten-specific tolerance (26).

Next to water, tea is the most popular beverage consumed world wide. In prior studies we showed that topical application

Abbreviations: APC, antigen-presenting cell; CHS, contact hypersensitivity; DAB, diaminobenzidine; DLN, draining lymph node; DNFB, 2,4-dinitrofluorobenzene; EGCG, (–)-epigallocatechin-3-gallate; FBS, fetal bovine serum; IL, interleukin.



(-)-Epigallocatechin-3-gallate

Fig. 1. Chemical structure of (–)-epigallocatechin-3-gallate, a green tea polyphenol.

of a polyphenolic fraction isolated from green tea before and after UV (2 kJ/m^2) irradiation to C3H/HeN mouse skin protects against UV-induced immunosuppression (27). This polyphenolic fraction is a mixture of four main epicatechin derivatives, i.e. (–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin gallate, (–)-epigallocatechin and (–)-epicatechin (28). EGCG, present in green tea, is the major and the most effective constituent among other polyphenolic constituents in affording protection against UV-induced carcinogenic and inflammatory effects (28,29). The chemical structure of EGCG is shown in Figure 1. Oral feeding of green tea extract as a sole source of drinking water to SKH-1 hairless mice has also been shown to protect against UV-induced suppression of CHS (30). Earlier, we have shown that blocking of UV-induced infiltrating leukocytes using anti-CD11b antibody (specific for monocytes/macrophages and neutrophils) or murine-specific soluble complement receptor type I reversed UV-induced immunosuppression and tolerance induction (31,32) in mice. These observations indicate the important role of infiltrating leukocytes in UV-induced immunosuppression and tolerance induction.

In the present study we have used a single low dose UVB (72 mJ/cm^2) exposure to C3H/HeN mouse skin. This UVB dose has previously been demonstrated to cause unresponsiveness and tolerance to 2,4-dinitrofluorobenzene (DNFB) when the sensitizer is applied to the UVB-exposed site, but not to a distant site (33). The aim of this study was to determine whether topical application of EGCG (3 mg/mouse) before UVB exposure will reverse UVB-induced tolerance induction and to determine the mechanisms involved in prevention of UVB-induced suppression of the CHS response and tolerance induction to a contact sensitizer by EGCG.

Materials and methods

Animals

Pathogen-free (MTV–) female C3H/HeN mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed in a pathogen-free barrier facility in accordance with current US Department of Agriculture and Department of Health and Human Services regulations and standards. Mice were kept four per cage and were acclimatized for 3–4 days before use, subject to a 12 h light/12 h dark cycle.

Antibodies and reagents

Monoclonal antibodies to mouse IL-10 (rat IgG2b, clone JES5-16E3), IL-12 (rat IgG1, clone C15.6) and CD11b were purchased from PharMingen (San Diego, CA). DNFB was purchased from Sigma Chemical Co. (St Louis, MO). Cytoscreen US™ mouse IL-10 and IL-12 ELISA kits were purchased from Biosource International (Camarillo, CA). Purified EGCG (99% pure) was obtained as a gift from Dr Yukihiko Hara (Mitsui Norin Co., Shizuoka, Japan). In all the experiments conducted in this study, EGCG was topically applied to the mouse skin 20 min before UVB exposure.

UVB irradiation

Forty-eight hours before UVB exposure, mice were shaved with electric clippers and Nair depilatory lotion was applied for 120–180 s. The period of Nair treatment remained constant in each group of animals as well as in each set of experiments. The non-UVB-exposed group of mice were also shaved and depilatory lotion was applied for the same time period to maintain the same treatment protocol. UVB irradiation was performed as described earlier (34). Briefly, the razor shaved and chemically depilated dorsal skin was exposed to UV irradiation from a band of six FS-40 fluorescent lamps from which UVB and UVC wavelengths not normally present in natural solar radiation were filtered out using Kodacel cellulose film (35). After filtration with a Kodacel film, the majority of the resulting wavelengths of UV radiation were in the UVB range (290–320 nm). Groups of mice were anesthetized by ketamine hydrochloride injection (Parke-Davis, Morris Plains, NJ) before UVB exposure to immobilize them so that a uniform and complete UVB dose could be delivered to the dorsal skin of the mice. UVB emission was monitored with an IL-443 phototherapy radiometer (International Light, Newburyport, MA) equipped with an IL SED 240 detector fitted with a W side angle quartz diffuser and a SC5 280 filter. A dose of 72 mJ/cm^2 UVB was delivered on the skin surface of each mouse. To maintain a similar treatment protocol and effects, non-UVB-exposed control groups of mice were also treated with Nair depilatory lotion and anesthetized with the same dose of ketamine hydrochloride.

All immunohistochemical analyses and ELISA assays were performed at 48 h after UVB irradiation of the mouse skin. This particular time point was chosen because the various end-points peaked at this time.

UVB induction of immunosuppression and tolerance induction

C3H/HeN mice were sensitized with DNFB by topical application of 25 μl of 0.5% DNFB in acetone:olive oil (4:1 v/v) onto the shaved, chemically depilated dorsal skin of either control mice (non-UVB-exposed), mice topically treated with EGCG (3 mg/mouse/200 μl acetone) alone 48 h before, mice exposed to UVB alone 48 h before or mice treated with EGCG (3 mg/mouse/200 μl acetone) plus UVB exposure 48 h before. The dorsal surface of the right ear was then challenged with 20 μl of 0.2% DNFB in acetone:olive oil (4:1 v/v) 5 days later. Twenty-four hours after challenge, ear skin thickness was measured using an engineer's micrometer (Mitutoyo, Tokyo, Japan) and compared with ear skin thickness just before the challenge. The degree of tolerance was determined by resensitizing the mice on razor shaved non-UVB-exposed abdominal wall skin with DNFB 48 h after the primary challenge. Five days after the secondary sensitization, mice were rechallenged with DNFB on the dorsal skin of the left ear. Ear swelling was measured immediately before and 24 h after rechallenge. Mice that received the same dose of DNFB but were not UVB irradiated served as a positive control, whereas the negative control mice were only ear challenged and received a sham irradiation procedure (shaving, chemical depilation, application of vehicle, anesthesia and restraint, but without UVB irradiation).

Preparation of epidermal cell suspension

Epidermal cell suspensions from control, UVB-exposed or EGCG + UVB-treated mice were prepared as described previously (31). Briefly, after removal of subcutaneous tissue, the skin was incubated in 0.25% trypsin (Sigma Chemical Co.) for 60 min at 37°C. The epidermis was separated from the dermis and dispersed into cell suspension in 0.025% DNase (Sigma Chemical Co.) in HBSS containing 10% heat-inactivated fetal bovine serum (FBS). The cell suspension was filtered through 50 μm nylon mesh (Tetko, Elmsford, NY) to obtain a single cell epidermal cell suspension.

Preparation of dermal cell suspension

Dermal cell suspensions from control, UVB-exposed or EGCG + UVB-treated mice were prepared as described previously (31). Briefly, skin was placed into a solution of dispase (Collaborative Research, Bedford, MA) overnight at 4°C. Dermis was separated from the epidermis and was placed into a digestion buffer of RPMI 1640 (Gibco BRL, Grand Island, NY) containing 10 mM HEPES (Irvine Scientific, Santa Ana, CA), 0.01% DNase, 1000 U/ml of hyaluronidase and collagenase (Sigma Chemical Co.) and digested at 37°C for 1 h. The digested dermal suspension was filtered through a 50 μm nylon mesh to obtain a single cell suspension.

Preparation of lymph node cell suspension

Inguinal lymph nodes were removed aseptically from UVB-exposed or EGCG + UVB-treated mice. Lymph nodes from non-UVB-exposed mice served as the control. The single cell suspensions were prepared by placing the lymph nodes in HBSS buffer containing 5% FBS. Connective tissues were teased out using fine sterile scissors to obtain lymph node cells from the lymph node capsule. The cell suspension was filtered through a 50 μm nylon mesh. The cells were washed three times in HBSS containing 5% FBS.

Immunohistochemical detection of CD11b+ cells in the skin

Immunostaining of CD11b was used as a cell surface marker of monocytes/macrophages and neutrophils. For immunostaining of CD11b+ cells, 6 μm thick sections of frozen skin were employed. After blocking non-specific staining using normal goat serum, sections were incubated with either rat anti-mouse CD11b antibody (Pharmingen) or RIgG2b isotype control. After washing in Tris–HCl buffer, pH 7.5, sections were incubated with biotinylated rabbit anti-rat IgG (Vector) and thereafter with peroxidase-labeled streptavidin. After washing in buffer, sections were incubated with diaminobenzidine (DAB) substrate solution (Kirkegaard & Perry, Gaithersburg, MD) and counterstained with methyl green.

Immunohistochemical detection of intracellular cytokines IL-10 and IL-12 in frozen sections

Six micron sections of frozen skin or DLN obtained from UVB-irradiated or EGCG + UVB-irradiated groups, as well as control mice (non-UVB-irradiated and EGCG alone-treated groups of mice) were fixed in ice-cold paraformaldehyde (4%) and immunostaining was performed as described earlier (36) with some modifications. Briefly, endogenous peroxidase was blocked with HBSS containing 0.1% saponin, 0.2 M sodium azide and 0.5% H_2O_2. For IL-10 and IL-12 cytokine staining, rat monoclonal anti-mouse IL-10 or IL-12 antibodies were diluted in HBSS + 0.1% saponin and incubated in a moist chamber at room temperature for 30 min. After washing in HBSS + 0.1% saponin, sections were incubated for 30 min in biotinylated rabbit anti-rat IgG (Vector) in HBSS + 0.1% saponin. After washing, sections were incubated with a solution of Vectastain Elite ABC (horseradish peroxidase) for 30 min. Vectastain Elite ABC solution was prepared according to the manufacturer's directions. After washing in HBSS alone, sections were incubated with DAB substrate solution (Kirkegaard & Perry) and counterstained with methyl green.

Images from immunostaining were obtained using a Zeiss Axiophot microscope and Kodak Ektachrome 160T film. These were scanned (Sprint Scan; Polaroid) and formatted as tiff images in Adobe Photoshop 3.0 and Microsoft Powerpoint in order to make the composite figures.

Measurement of cytokines IL-10 and IL-12 by ELISA

Single cell suspensions obtained either from epidermis, dermis or DLN were incubated at 37°C for 24 h in RPMI 1640 containing 10% FBS. Cells were centrifuged and supernatants were collected and filtered through a 0.2 μm cellulose acetate membrane filter to determine IL-10 or IL-12 protein by ELISA (Biosource International, Camarillo, CA) following the manufacturer's directions. The sensitivity of the mouse IL-10 ELISA was <0.2 pg/ml, while that of IL-12 was <2 pg/ml.

Statistical analysis

Student's *t*-test was employed to determine statistical significance between treated and untreated groups.

Results

Skin treatment with EGCG before UVB exposure reverses UVB-induced immunosuppression

Skin treatment with EGCG on non-UVB-exposed, control mice did not affect the ability of the mice to generate a contact sensitivity response to DNFB (Figure 2, third bar from the top) compared with DNFB-treatment alone (positive control, second bar from the top). Mice with UVB exposure alone were unable to be sensitized, either through the UVB-exposed site (Figure 2, left, fourth bar from the top) or through normal, non-UVB-exposed abdominal skin after the primary sensitization through UVB-exposed skin (tolerance, Figure 2, right, fourth bar from the top). However, skin treatment with EGCG before UVB exposure showed the ability of the mice to develop a contact sensitivity response (Figure 2, left, fifth bar from the top) concomitant with partial prevention of tolerance induction (Figure 2, right, fifth bar from the top). Thus, skin treatment with EGCG of non-UVB-irradiated mice does not interfere with the capability of the mice to generate a contact sensitivity response, but does restore the ability of UVB-irradiated mice to induce a primary contact sensitivity response as well as partially blocking tolerance induction.

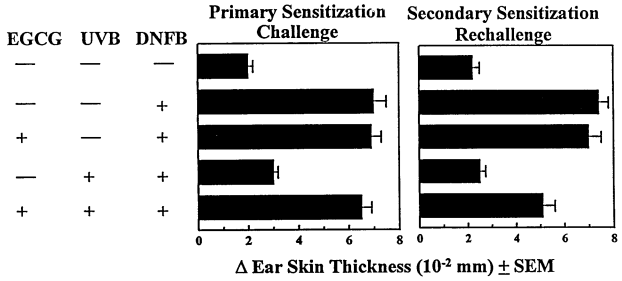


Fig. 2. Skin treatment with EGCG before UVB exposure reverses UVB-induced immunosuppression. (Left) The ear swelling response to primary sensitization through UVB-irradiated dorsal skin and challenge to the right ear. (Right) The response to secondary sensitization through normal non-UVB-irradiated abdominal skin and rechallenge to the left ear. UVB-exposed mice were unable to be sensitized, either through the UVB-exposed site (left, fourth bar from the top) or through normal, non-UVB-exposed abdominal skin after the primary sensitization through UVB-exposed skin (tolerance, right, fourth bar from the top). However, EGCG treatment before UVB exposure showed the ability to develop a CHS response (left, fifth bar from the top) concomitant with partial reversal of tolerance induction (right, fifth bar from the top). Data are expressed as means ± SEM change in ear skin thickness between pre-challenge and 24 h post-challenge measurements. Similar results were obtained in two repeat experiments. The details are provided in Materials and methods.

Skin treatment with EGCG before UVB exposure reduces both the numbers of infiltrating CD11b+ cells and damage to the UVB-irradiated epidermis

Forty-eight hours after UVB irradiation of the skin, infiltrating leukocytes (predominately monocytes/macrophages and neutrophils) were present in higher numbers in the skin, particularly in the dermis (Figure 3, middle), compared with control, non-UVB-exposed skin (Figure 3, left). It has been shown that leukocytes (monocytes/macrophages and neutrophils) infiltrating into UV-irradiated skin play a critical role in UVB-induced immunosuppression and tolerance induction (31,37). In our present study we found that application of EGCG before UVB exposure significantly reduced the number of infiltrating leukocytes into the skin (Figure 3, right), when compared with UVB alone-exposed skin (Figure 3, middle). Further, immunostaining of CD11b was used to determine whether skin application of EGCG was able to reduce the number of infiltrating leukocytes induced by UVB exposure. In control skin, monocytes/macrophages and neutrophils are clearly stained with the anti-CD11b antibody (Figure 3). Forty-eight hours after UVB exposure the numbers of CD11b+ cells were markedly increased in comparison with control, non-UVB-exposed skin. Treatment with EGCG before UVB exposure significantly reduced the number of UVB-induced CD11b+ cells in the skin (Figure 3), whereas application of EGCG alone to the mouse skin did not seem to alter the constitutive pattern of the cells when compared with the normal skin of the mice and also did not induce infiltration (data not shown). The CD11b+ cells in different treatment groups were counted at random using an ocular micrometer grid under a Zeiss Axiophot microscope with 200× magnification corresponding to a 0.0625 mm^2 area. These CD11b+ cells accounted for 12 ± 4, 28 ± 5 and 17 ± 5 cells, respectively, in interfollicular regions of the dermis of the control (Figure 3, left), UVB-exposed (Figure 3, middle) and EGCG + UVB-treated (Figure 3, right) mouse skin. Thus treatment with EGCG before UVB exposure of the skin resulted in a 69% ($P < 0.001$) reduction in UVB-induced infiltration of CD11b+ cells. After 48 h UVB irradiation a fraction of these CD11b+

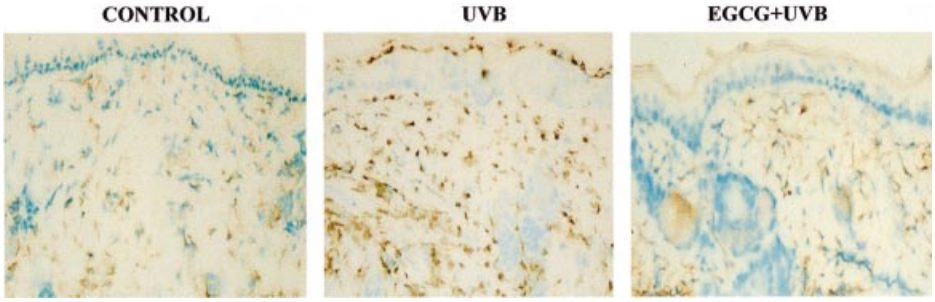


Fig. 3. Immunohistochemical detection of CD11b+ cells in mouse skin. Six micron thick frozen skin sections were stained with rat anti-mouse CD11b monoclonal antibody after fixation in cold acetone. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide in Tris–HCl buffer. Biotinylated rabbit anti-rat IgG was used as a secondary antibody; thereafter sections were incubated with peroxidase-labeled streptavidin. Immunostaining of CD11b+ cells is shown in brown and is representative of three independent experiments. Magnification ×200.

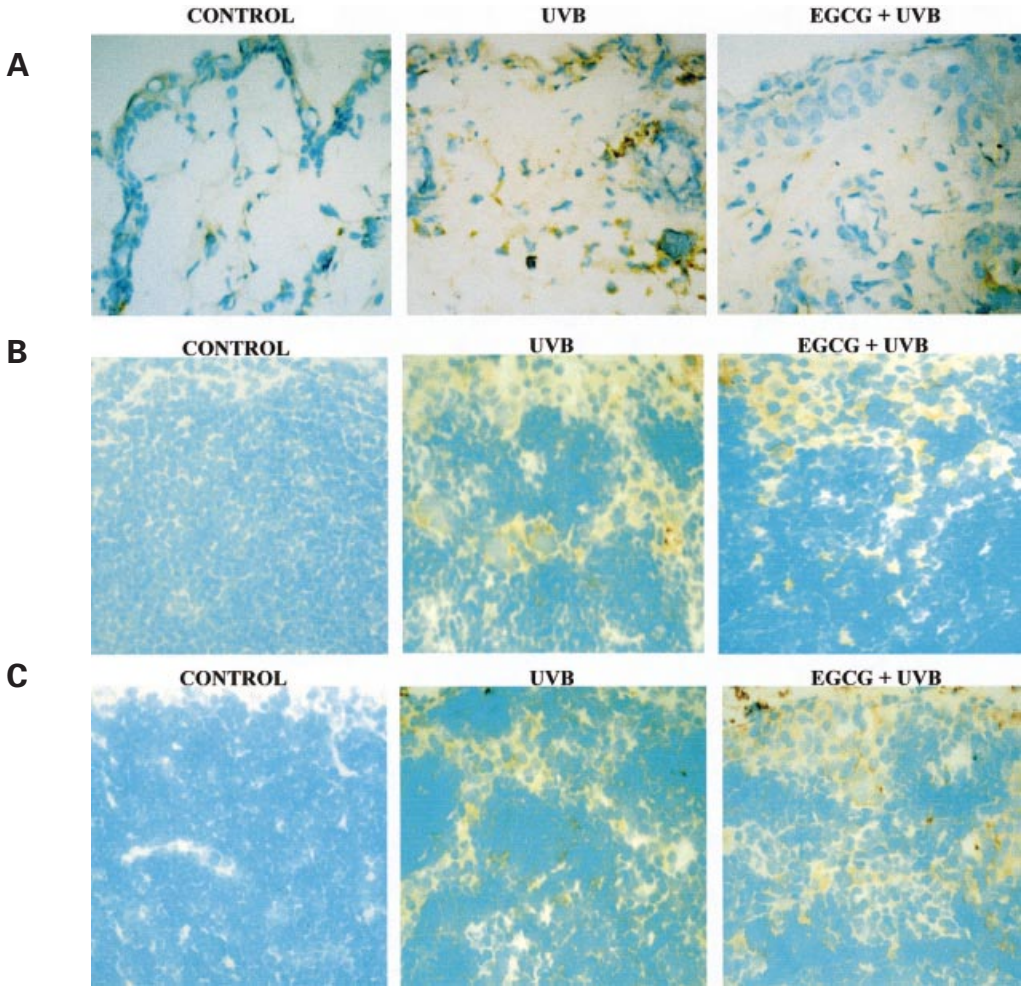


Fig. 4. (**A**) Immunohistochemical detection of IL-10+ cells in mouse skin. Six micron thick frozen skin sections were fixed in cold 4% paraformaldehyde. For immunohistochemical detection of IL-10-producing cells intracellular staining was performed using rat anti-mouse IL-10 monoclonal antibody, which was diluted in HBSS buffer containing 0.1% saponin. After washing, sections were incubated in biotinylated rabbit anti-rat IgG and subsequently in horseradish peroxidase Vectastain Elite ABC reagent solution. Immunostaining of IL-10+ cells is shown in brown. The immunostaining shown is representative of three independent experiments. Magnification ×400. (**B**) Immunohistochemical detection of IL-10+ cells in DLNs of mice. Six micron thick frozen lymph node sections were fixed in cold 4% paraformaldehyde. For immunohistochemical detection of IL-10-producing cells intracellular staining was performed using rat anti-mouse IL-10 monoclonal antibody, which was diluted in HBSS buffer containing 0.1% saponin. After washing, sections were incubated in biotinylated rabbit anti-rat IgG and subsequently in horseradish peroxidase Vectastain Elite ABC reagent solution. Immunostaining of IL-10+ cells is shown in brown. The immunostaining shown is representative of three independent experiments. Magnification ×400. (**C**) Immunohistochemical detection of IL-12+ cells in DLNs of mice. Six micron thick frozen lymph node sections were fixed in cold 4% paraformaldehyde. For immunohistochemical detection of IL-12-producing cells intracellular staining was performed using rat anti-mouse IL-12 monoclonal antibody, which was diluted in HBSS buffer containing 0.1% saponin. After washing, sections were incubated in biotinylated rabbit anti-rat IgG and subsequently in horseradish peroxidase Vectastain Elite ABC reagent solution. Immunostaining of IL-12+ cells is shown in brown. The immunostaining shown is representative of three independent experiments. Magnification ×400.

cells were able to reach the upper part of the epidermis, as shown in Figure 3 (middle). In the UVB-irradiated skin (Figures 3 and 4A), disruption appears as damage to the epidermis and loss of normal keratinocyte stratification. This suggests the possibility that infiltrating leukocytes are causing a secondary injury to the UVB-exposed epidermis that results in keratinocyte disassociation and a breakdown in the structure of the UVB-exposed epidermis. EGCG treatment before UVB exposure appears to protect against UVB-induced damage to the epidermis.

Skin treatment with EGCG before UVB exposure reduces the number of UVB-induced IL-10-producing cells and IL-10 protein production in skin

Intracellular immunostaining of IL-10 showed that UVB exposure induces IL-10-producing cells in mouse skin (Figure 4A), but treatment with EGCG before UVB exposure resulted in decreased numbers of IL-10-producing cells both in epidermis and dermis. Most of the IL-10+ cells, which appeared to be infiltrating leukocytes, were observed in the lower part of the papillary dermis, but a few IL-10+ cells were also observed in the epidermis, which appeared to be infiltrating cells following UVB exposure. A few IL-10+ cells were also noted in the dermis of control skin (Figure 4A). IL-10 protein quantitation by ELISA (pg/million cells) also confirms that UVB exposure stimulates IL-10 protein synthesis in epidermal and dermal cells (Figure 5A). Skin treatment with EGCG before UVB exposure resulted in 87 and 83% ($n = 3$, $P < 0.005$) reductions in IL-10 protein concentration in epidermal and dermal cells, respectively, in comparison with UVB alone. The decrease in UVB-induced IL-10-producing cells and protein production in both epidermal and dermal cells by EGCG may be associated with blocking of infiltrating leukocytes after UVB exposure. Treatment with EGCG alone on the dorsal skin of the mice does not appear to increase or decrease the number of IL-10+ cells (data not shown). Thus, it appears that infiltrating leukocytes are the major source of IL-10 production in UVB-exposed skin. It is also evident from Figure 4A that exposure of skin to UVB damages the epidermis, while application of EGCG protects against UVB-induced damage as well as maintaining the structure of the epidermis. Because of this, the thickness of UVB-exposed mouse skin (Figure 4A, middle) appears thinner than EGCG + UVB-treated mouse skin.

Skin treatment with EGCG before UVB exposure reduces the number of UVB-induced IL-10-producing cells and IL-10 protein production in DLN

Lymph nodes are an important part of the immune system because they provide the cellular scaffolding necessary for interactions of immune cells. After skin exposure to UVB, APC migrate to regional lymph nodes and activate T cells to stimulate immune responses. Immunohistochemical staining showed that UVB exposure of skin increases the number of IL-10-producing cells in the DLN, in an area ranging from the subcapsular sinus to the paracortical region of the lymph nodes, including interfollicular areas, which are specific sites of T cell localization (Figure 4B). DLN cells from untreated mice (control) do not produce IL-10, as evidenced by the absence of IL-10 immunostaining (Figure 4B) as well as IL-10 protein quantitation by ELISA (Figure 5B). DLN cells from EGCG alone-treated mice also do not show the presence of IL-10 immunostaining as well as IL-10 protein by ELISA (data not shown). Skin treatment with EGCG before UVB exposure results in reduced numbers of IL-10+ cells (Figure 4B) as well as decreased IL-10 protein production in DLN (60% reduction, $n = 3$, $P < 0.005$) as compared with UVB alone-exposed mice (Figure 5B).

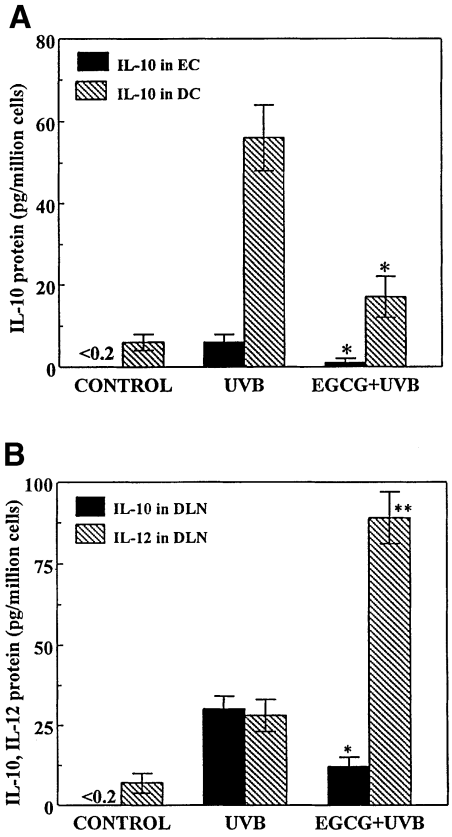


Fig. 5. (**A**) Quantitative determination of IL-10 protein in epidermal cells (EC) and dermal cells (DC) of mouse skin by ELISA. Quantitative estimation of IL-10 protein was performed using ELISA in supernatants of single cell suspensions from epidermis and dermis. Details are provided in Materials and methods. Quantities of IL-10 protein production are expressed as pg/million cells and represent means ± SEM. $n = 3$ repeated experiments on four mice (total 16 inguinal lymph nodes per group) in each group per experiment. *Highly significant reduction in IL-10 versus UVB alone, $P < 0.005$. (**B**) Quantitative determination of IL-10 and IL-12 proteins in DLN cells of mice by ELISA. Quantitative estimations of IL-10 and IL-12 proteins were performed by ELISA in supernatants of a single cell suspension from DLN. Details are provided in Materials and methods. Quantities of IL-10 and IL-12 proteins are expressed as pg/million cells and represent means ± SEM. $n = 3$ repeated experiments on four mice (total 16 inguinal lymph nodes per group) in each group per experiment. *Highly significant reduction in IL-10 protein production versus UVB exposure alone, $P < 0.005$. **Highly significant increase in IL-12 protein production versus UVB exposure alone, $P < 0.0005$.

Skin treatment with EGCG before UVB exposure increases the number of IL-12-producing cells and IL-12 protein production in DLN

As is evident from immunostaining, skin exposure to UVB induces IL-12+ cells in the DLN (Figure 4C). Skin treatment with EGCG before UVB exposure further increases the number of IL-12+ cells in DLN in comparison with UVB alone-exposed mice (Figure 4C). Quantitation of IL-12 protein using ELISA indicates that skin exposure to UVB results in increased production of IL-12 in DLN (28 pg/million cells) and skin treatment with EGCG before UVB exposure further markedly increases IL-12 protein production (89 pg/million cells) in DLN, as shown in Figure 5B. Thus, EGCG application before

UVB exposure induced a 4-fold increase in IL-12 production in comparison with increased production of IL-12 by UVB alone. Unlike IL-10, IL-12 protein was detectable (7 pg/million cells) in control DLN cells. Similar to the control group of mice, in the EGCG only treatment group, IL-12 immunostaining was not detectable but IL-12 protein was detectable by ELISA in DLN (data not shown). It is important to mention that we were not able to detect IL-12+ cells in skin biopsies of any of the treatment groups using immunohistochemistry. This information is consistent with the current knowledge of IL-12 in the skin (38).

Induced production of IL-12 in DLN after UVB exposure of the skin may be due to cellular migration from the skin to DLN. In the case of EGCG treatment before UVB exposure, a marked increase in IL-12 was observed in comparison with UVB alone. It appears that EGCG application prevents cellular damage or cell death caused by UVB exposure of the skin, therefore, the number of cells migrating from the skin to DLN may be higher in EGCG + UVB-exposed skin in comparison with UVB alone-exposed skin. Thus, our data indicate that skin treatment with EGCG before UVB exposure reduces UVB-induced IL-10 (Figures 4B and 5B) while increasing IL-12 production in DLN in comparison with UVB alone (Figures 4C and 5B).

Discussion

Our CHS data clearly demonstrate that skin treatment with EGCG before a single low dose UVB exposure to C3H/HeN mice almost completely prevents UVB-induced immunosuppression and partially blocks tolerance induction. The mechanisms involved in immunosuppression induced by UV exposure differ greatly. There are several views to describe a crucial *in vivo* role for IL-12 in the induction of a CHS response. CHS appears to be a Th1-mediated immune response (39) and Langerhans cells, critical APC in the induction phase of CHS (8), were described as an additional source of IL-12. The concept of sensitization is that haptens, when applied to the skin, bind to peptides attached to the MHC molecules on epidermal Langerhans cells as the APC of the epidermis (40). These cells migrate to the regional DLN to initiate sensitization. Therefore, we were interested to determine whether UV-induced immune suppression and tolerance induction results through infiltrating CD11b+ cells and up-regulation of IL-10 production and/or down-regulation of IL-12. Another point of interest was to determine whether topical application of EGCG before UVB injury results in blocking of infiltrating CD11b+ cells, down-regulating IL-10 and up-regulating IL-12 in skin and/or DLN, thereby preventing UV-induced immunosuppression and tolerance induction.

Skin treatment with EGCG does prevent UVB-induced immunosuppression and tolerance induction and this prevention is associated with a reduction in the number of CD11b+ monocytes/macrophages and neutrophils infiltrating into UVB-irradiated skin. The infiltrating CD11b+ cells could provide a second hit, via reactive oxygen species, to the UV-damaged epidermis that results in keratinocyte disassociation and further breakdown in the structure of the UVB-exposed epidermis. It has previously been shown that blocking of infiltrating leukocytes using anti-CD11b antibody and also using soluble complement receptor type I blocks UV-induced immunosuppression and tolerance induction and also prevents further damage to UV-irradiated epidermis in mice (31,32).

We feel that in the skin the major IL-10-producing cells may not be the constitutive APC but infiltrating leukocytes (Figure 4A). A number of studies are available showing the immunomodulatory effects of IL-10 *in vitro*. They provide convincing evidence that IL-10 inhibits antigen presentation (41,42) and secretion of cytokines by macrophages (43,44). Some reports are available showing the *in vivo* effects of IL-10 in T cell-mediated reactions, such as i.p. administration of IL-10 to mice inhibiting their ability to be sensitized to trinitrophenyl-coupled spleen cells for a delayed type hypersensitivity response (18). These investigators showed that i.p. injection of IL-10 into sensitized mice 24 h before challenge resulted in a significant suppression of the ear swelling response, suggesting that IL-10 is able to block the effector phase, but not the induction phase, of CHS *in vivo*. Further, administration of neutralizing antibodies to IL-10 largely, but not totally, inhibited the ability of UV irradiation to suppress sensitization to alloantigens (19). Intraperitoneal injection of anti-IL-10 antibody into mice prevented UV-induced tolerance induction (20). In agreement with these observations, our data demonstrate that skin treatment with EGCG before UVB exposure results in a decreased amount of IL-10 production in skin as well as in DLN, suggesting a possible mechanism by which EGCG prevents UVB-induced immunosuppression in mice.

In UV-exposed epidermis, the number of IL-10-producing cells (Figure 4A) and its production (Figure 5A) are higher than in normal and EGCG + UVB-treated skin. The higher production of IL-10 in epidermis may send a tolerizing signal to epidermal Langerhans cells (45). Skin treatment with EGCG significantly reduces the number of IL-10-producing cells and its production in epidermis (Figures 4A and 5A), suggesting a possible mechanism of protection against UVB-induced tolerance induction. Moreover, EGCG shows a peak of absorption spectra at near 270–275 nm in UV range. It may be possible that EGCG functions as a sunscreen for certain UV radiation wavelengths, thus protecting the antigen-presenting capacity of skin APC against UV irradiation and, eventually, protecting against UV-induced immunosuppression.

IL-12 regulates the growth and function of T cells (22) and, especially, the development of Th1-type cells by stimulating the production of IFN-γ (46–48). It has been demonstrated that i.p. injection of rIL-12 prevents UV-induced immunosuppression (25) and overcomes UV-induced hapten-specific tolerance (26). Furthermore, tolerance of contact sensitization was induced by IL-10 administration in the induction phase, suggesting a role of Th2-type cytokines in contact sensitization tolerance (49). These studies imply that a cytokine imbalance between Th1 versus Th2 cells may be responsible for development of UVB-induced tolerance in contact sensitization and that IL-12 could prevent the induction of tolerance by antagonizing Th2 cytokines. In our chemopreventive model, where EGCG is used before UVB exposure, EGCG application seems capable of tilting the immune response in favor of the development of Th1-type cells and Th1 cytokine production. Therefore, this cytokine shift by EGCG may be the mechanism of action responsible for reversing UVB-induced immunosuppression and tolerance induction in mice. It is evident that radiolabeled EGCG orally administered to mice is distributed to various organs of the body including the skin (50). The presence of EGCG in the skin after administration of green tea as a sole source of drinking water to mice may be the reason for protection against UVB-induced photocarcinogenesis (51).

Additionally, topical application of EGCG (10 or 50 mg EGCG/mouse) before UV exposure prevents photocarcinogenesis in BALB/cAnN Hsd mice (52). It is tempting to suggest that protection against UVB-induced immunosuppression by green tea may be associated with protection against UVB-induced photocarcinogenesis.

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