Green Tea Polyphenol-Induced Epidermal Keratinocyte Differentiation Is Associated with Coordinated Expression of p57/KIP2 and Caspase 14

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ABSTRACT

Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, exerts chemopreventive effects by selectively inducing apoptosis in tumor cells. In contrast, EGCG accelerates terminal differentiation in normal human epidermal keratinocytes (NHEK) mediated partially by up-regulation of p57/KIP2, a cyclin-dependent kinase inhibitor that confers growth arrest and differentiation. However, it is unclear if EGCG modulates caspase 14, a unique regulator of epithelial cell terminal differentiation associated with cornification. Here, we examined the effect of EGCG on caspase 14 expression in NHEK and correlated the protein and mRNA expression of p57/KIP2 in either normal keratinocytes or p57/KIP2-expressing tumor cells (OSC2, an oral squamous cell carcinoma cell line). Additionally, paraffin-embedded normal and untreated psoriatic (aberrant keratinization) skin sections from humans were assessed for caspase 14 by immunohistochemistry. In NHEK, EGCG induced the expression of caspase 14 mRNA and protein levels within a 24-h period. The expression of p57/KIP2 in OSC2 cells was adequate to induce caspase 14 in the absence of EGCG; this induction of caspase 14 was down-regulated by transforming growth factor-β1. In human psoriatic skin samples, caspase 14 staining in the upper epidermis was reduced, especially in nuclear areas. These results suggest that, in addition to p57/KIP2, EGCG-induced terminal differentiation of epidermal keratinocytes involves up-regulation of caspase 14. Further understanding of how EGCG modulates cellular differentiation may be useful in developing green tea preparations for selected clinical applications.

Unique characteristics of green tea polyphenols include their ability to induce growth arrest and apoptosis in tumor cells, especially in epithelial-type cells (Hsu et al., 2002b; Adhami et al., 2003), as well as protecting normal epithelial cells from carcinogens (Mukhtar and Ahmad, 2000; Katlayar, 2003). Among the four major polyphenols present in green tea, (−)-epigallocatechin-3-gallate (EGCG) is the most abundant (Miyazawa, 2000).

This study was supported in part by a grant from the Medical College of Georgia Research Institute, a National Cancer Institute Grant R21 CA097258-01A1, and funding through the Department of Oral Biology and Maxillofacial Pathology, School of Dentistry, Medical College of Georgia (to S.H.).

ABBREVIATIONS: EGCG, (−)-epigallocatechin-3-gallate; NHEK, normal human epidermal keratinocytes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TGF-β1, transforming growth factor-β1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein.

We previously reported that green tea polyphenols, EGCG in particular, activate a pathway for cell differentiation in normal human epidermal keratinocytes (NHEK). Unlike epithelial-derived tumor cells, NHEK undergo an accelerated differentiation that is associated with p57/KIP2 induction when exposed to EGCG (Hsu et al., 2001, 2002a, 2003a). The p57/KIP2 gene product is a p53-independent G1 cyclin/cyclin-dependent kinase inhibitory protein (Lee et al., 1995); the C terminus contains a binding domain for proliferating cell nuclear antigen (Watanabe et al., 1998). Embryonic development in mice requires p57/KIP2 expression, lack of which causes early postnatal death and growth retardation (Takahashi et al., 2000). Conversely, in continuously dividing hu-
man intestinal cell models, elevations of p57/KIP2 are associated with differentiation (Deschenes et al., 2001). Whereas other cyclin-dependent kinase inhibitors such as p16, p21, and p27 are proapoptotic (Opalka et al., 2002), p57/KIP2 confers growth arrest, differentiation, and cell survival. In human epidermis, the stratum corneum consists of anucleated keratinocytes with cross-linked proteins and lipids that generate a mechanical barrier (Madison, 2003). This barrier formation relies on the ciliation of epidermal keratinocytes, which undergo growth arrest, terminal differentiation, and an epidermal-specific apoptosis referred to by Nickoloff as “planned cell death” (Nickoloff et al., 2002). Abnormalities in any of these programmed features may manifest in skin disorders such as psoriasis or cancer. However, the mechanisms triggering proliferating keratinocytes to enter planned cell death are not completely understood (Martinez et al., 1999; Alonso and Fuchs, 2003).

Caspase 14, identified in 1998 first in mice and later humans (Ahmad et al., 1998), is expressed only in epithelial tissues, especially the differentiating regions of the outer epidermis. Unlike the other caspases, caspase 14 is not involved in the typical apoptotic cascade but is associated with terminal differentiation of NHEK and barrier formation (Eckhart et al., 2000b; Lippens et al., 2000). In addition to the epidermis, caspase 14 expression also is found in the choroid plexus, the retinal pigment epithelium, and thymic Hassall’s bodies, tissues that function as barriers (Lippens et al., 2003). A series of elegant experiments showed that epidermal induction of caspase 14 at the transcriptional level occurs during stratum corneum formation, whereas inhibition of cell differentiation results in diminished caspase 14 expression (Eckhart et al., 2000a; Rendl et al., 2002). Thus, caspase 14 is thought to specifically modulate epidermal differentiation, possibly signaling terminal differentiation and cornification of the epidermis (Mikolajczyk et al., 2004). In psoriasis, whereby rapidly migrating keratinocytes result in abnormal cornification, caspase 14 expression is altered (Lippens et al., 2000).

It is unknown whether caspase 14 is involved in EGCG-induced keratinocyte differentiation or EGCG-induced p57/KIP2 expression. We hypothesized that EGCG activates a differentiation pathway in normal epidermal keratinocytes and an apoptotic pathway in tumor cells (Hsu et al., 2003c). Here, we investigated the relationship between EGCG-induced p57/KIP2 and caspase 14 expression using in vitro models of NHEK, an oral squamous cell carcinoma line (OSC2), and subclones derived from OSC2 with stable expression of p57/KIP2. Expression patterns of caspase 14 in normal and psoriatic human skin samples were also examined.

**Materials and Methods**

**Chemicals and Antibodies.** EGCG and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-human caspase 14 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-human p57/KIP2 antibody was purchased from BD Biosciences PharMingen (San Diego, CA).

**Cell Lines and Cell Culture.** NHEK were purchased from Cambrex Corporation (East Rutherford, NJ) and subcultured in KGM-2 provided by the manufacturer. Subculture of the NHEK was performed by detaching the cells in 0.25% trypsin and transfer into new tissue culture flasks. The human oral squamous cell carcinoma lines OSC2 and the subclones (G6, S1, S2, S3, S4, and S5) were maintained in DMEM/Ham’s F-12 medium with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 5 µg/ml hydrocortisone.

**Immunocytochemistry of NHEK.** To evaluate the intracellular distribution patterns of caspase 14 in NHEK, exponentially growing NHEK were seeded in 8-well chamber slides (Nalge Nunc International, Naperville, IL) 12 h prior to EGCG treatment. At the end of a 24-h treatment, the slides were washed with PBS and fixed in ice-cold 4% paraformaldehyde solution for 10 min. Then, 3% hydrogen peroxide solution and normal goat serum were applied to block endogenous peroxidase activity and nonspecific binding. The caspase 14 antibody was applied for 1 h at 37°C at a dilution of 1:50. A streptavidin detection technique (Biogenex, San Ramon, CA) was used with 3-amin-9-ethylcarbazole as chromogen. Mayer’s hematoxylin was used as a counterstain. Negative control slides consisted of cells treated with 1% diluted normal goat serum instead of primary antibody.

**Human Skin Sample Collection and Processing for Caspase 14 Expression.** Under an approved human use protocol, 4-mm punch biopsies of normal (n = 3) and untreated psoriatic skin samples (n = 6) were obtained from different patients who provided written, informed consent. Samples were fixed in 10% neutral-buffered formalin, paraffin embedded, sectioned at 5 μm, and immunostained for caspase 14 presence by immunohistochemistry using a modified avidin-biotin-peroxidase technique described previously (Hsu et al., 2003a). The paraffin sections were deparaffinized in xylene (Sigma-Aldrich) and dehydrated in a descending series of ethanol to water. Endogenous peroxidase activity was blocked by a 5-min incubation in 3% H2O2. Nonspecific binding of the antibody to tissue sections was blocked by incubation for 1 h in 10 mg/ml bovine serum albumin. Rabbit anti-caspase 14 (Santa Cruz Biotechnology, Inc.) at a dilution of 1:50 was applied to the sections for 1 h. Control sections were treated with normal goat serum instead of the anti-caspase 14 antibody. After washing in PBS, a 1:200 dilution of the secondary biotin-conjugated goat anti-rabbit immunoglobulin antibody (Vector Laboratories, Burlingame, CA) was applied for 30 min. The sections were washed in PBS and then incubated for 30 min in an avidin-biotin-peroxidase complex reagent (ABC reagent; Vector Laboratories). Following three additional PBS washes, the peroxidase molecule in the immobilized avidin-peroxidase complex was used to reduce H2O2 in the presence of Vector NovaRED substrate (Vector Laboratories) to produce a red reaction product. In addition, sections were counterstained with Mayer’s hematoxylin as an aid to morphological identification. After staining, tissue sections were dehydrated in ascending concentrations of ethanol to xylene and coverslipped with Cytoseal XYL (Stephens Scientific, Riverside, NJ).

**Total RNA Extraction and Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** To determine the temporal sequence of EGCG-activated p57/KIP2 and caspase 14 expression, total RNA samples were obtained from cells incubated with 50 μM EGCG for 0, 0.5, 2, 6, 8, 12, 20, and 24 h. To determine whether expression of p57/KIP2 in OSC2 cells up-regulated caspase 14 levels, total RNA was collected from five p57/KIP2-expressing subclones (S1–S5) along with the parental cell line OSC2 and a mock-transfected cell line G6. To determine whether p57/KIP2 mRNA was reduced by TGF-β1, an inhibitor of gene expression and proteolytic degrader of p57/KIP2 (Uran et al., 1999, Nishimori et al., 2001), the S subclones, along with the OSC2 and G6 cell lines, were treated with 1 ng/ml TGF-β1 for 24 h followed by RNA preparation. The mRNA species were analyzed by semiquantitative RT-PCR using primers specific for p57/KIP2, caspase 14, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Total RNA was extracted using an RNeasy total RNA isolation system (QIAGEN, Valencia, CA) and quantified using the Ribogreen Assay (Molecular Probes, Eugene, OR). The extracted RNA (1 µg) was...
added to 20 μl of reverse transcription buffer and 2.5 U MuLV reverse transcriptase (GeneAmp RNA PCR kit; Applied Biosystems, Forest City, CA). This mixture was incubated at 42°C for 15 min and at 99°C for 5 min. The primers for caspase 14 were designed according to the GeneBank sequence for caspase 14 (accession no. NM012114): sense, 5'-ATATGATATGCATTGCGCCG-3' and antisense, 5'-TACCATGGAATATACAGTGCAAA-3'. The PCR was performed on the upper layers (Fig. 1D). The immunohistochemistry result showed that caspase 14 staining appearing to involve the cytosol and nuclei.

**Results**

**Caspase 14 Expression in EGCG-Treated NHEK.** The immunocytochemistry result demonstrated that caspase 14 protein was almost undetectable without EGCG exposure in subconfluent control samples of NHEK (Fig. 1A). Cells incubated with EGCG for 24 h showed large amounts of caspase 14 staining appearing to involve the cytosol and nuclei (Fig. 1B).

**Caspase 14 Expression in Normal and Psoriatic Skin.** The immunohistochemistry result showed that normal human epidermis exhibited minimal staining in the basal layers and intense homogeneous caspase 14 immunostaining in the upper layers, including nuclear staining (Fig. 1C). In contrast, the psoriasis samples showed a reduction in epidermal caspase 14 immunostaining, especially in the nuclei of the upper layers (Fig. 1D).

**EGCG-Modulated Expression of p57/KIP2 and Caspase 14 in NHEK.** In preconfluent NHEK culture, EGCG induced p57/KIP2 expression at 2 h, but the largest amount of p57/KIP2 mRNA (approximately 5-fold over the control, normalized to GAPDH) was observed at 6 h, followed by a gradual decline to approximately 3-fold over the control level at 24 h (Fig. 2A). EGCG also increased caspase 14 mRNA within 30 min (Fig. 2A). The RT-PCR products increased 14- to 17-fold over the control level, normalized to GAPDH, from 0.5 to 8 h and climbed to 25-fold of control levels by 12 h before declining to 18-fold over the control level at 24 h (Fig. 2A). The p57/KIP2 induction was transient since p57/KIP2 mRNA declined gradually after it peaked, whereas EGCG-induced caspase 14 mRNA was persistent compared with p57/KIP2 induction.

**EGCG Modulated Protein Levels of p57/KIP2 and Caspase 14 in NHEK.** p57/KIP2 protein levels were significantly elevated at 4 h and peaked at 8 h, a 14-fold increase relative to control levels normalized to actin (Fig. 2B). Levels of p57/KIP2 protein remained relatively stable during the
later hours and declined to approximately 7-fold of control levels at 24 h. Protein levels of caspase 14 gradually increased from 4 h and peaked at 20 h for an increase of 5-fold compared with control, normalized to actin (Fig. 2B). EGCG-induced p57/KIP2 protein accumulation was an earlier event compared with that of caspase 14.

Effects of Retrovirus-Mediated p57/KIP2 Expression on S Subclones in Response to EGCG. Subclones of OSC2 cells expressing either p57/KIP2 (S1–S5) or green fluorescent protein (G6) were established and maintained in DMEM/F-12 culture medium. OSC2 cells and G6 cells exhibited similar degrees of dose-dependent loss of cell viability, whereas p57/KIP2-expressing S1, S3, and S4 cells did not exhibit cytotoxicity in response to 100 μM EGCG; S1–S4 cells also showed a lower degree of cytotoxicity to 200 μM EGCG than OSC2 and G6 cells (Fig. 3A). In fact, S1, S3, and S4 cells...
treated with 100 μM EGCG exhibited increased viability resembling that of aged NHEK exposed to EGCG (Hsu et al., 2003a). The S5 cells were similar to OSC2 and G6 cells in their response to EGCG exposure (Fig. 3A). However, S5 cells exhibited a much lower proliferation rate; the cell number of S5 did not increase during the 48-h period, whereas OSC2 and G6 cell numbers increased more than 3-fold. S1–S4 cells exhibited reduced growth rates in comparison to the parental cells or G6 cells, but greater rate than S5 cells (Fig. 3B).

p57/KIP2 Expression in OSC2 Cells Activated Expression of Caspase 14. The semiquantitative RT-PCR amplification of total RNA samples demonstrated that both OSC2 and G6 cells expressed only minimal levels of p57/KIP2 message, whereas S1, S2, S3, and S5 expressed higher levels (approximately 200%) of p57/KIP2 message than OSC2 or G6, and S4 cells expressed considerable (approximately 150%) p57/KIP2 message (Fig. 4A). When caspase 14 mRNA from these cell lines was amplified by semiquantitative RT-PCR, OSC2 and G6 exhibited only background levels of caspase 14 mRNA. In contrast, the S subclones expressed higher levels of caspase 14 mRNA, indicating activation of caspase 14 gene expression (Fig. 4A).

Discussion

EGCG induces production of involucrin in epidermal keratinocytes, an important protein in keratinocyte differentiation (Balasubramanian et al., 2002). We previously demonstrated that EGCG accelerated NHEK differentiation with increased keratin 1 and filaggrin expression and activation of transglutaminase (Hsu et al., 2003a). Here, we report that EGCG induces caspase 14, a recently described putative regulator for keratinocyte terminal differentiation, within a 24-h period with evidence suggesting it may be induced independently by p57/KIP2, a well established regulator of cell growth and differentiation (Figs. 1 and 2). We also found that epidermal cells in psoriasis, a disease characterized by aberrant keratinization, exhibit reduced caspase 14 expression, particularly in the nuclear regions of the cells (Fig. 1). This observation provided an in vivo human correlate for the relationship between caspase 14 expression and terminal differentiation. Together with our in vitro data, this suggested an alternative approach would be available to psoriasis treatment besides currently licensed modalities, most of which are immunosuppressive with untoward side effects. Collectively, the data demonstrate that EGCG enabled NHEK to accelerate terminal differentiation, marked by a coordinated activation and expression of p57/KIP2 and caspase 14. Since caspase 14 is strictly associated with NHEK terminal differentiation and skin barrier formation (Mikolajczyk et al., 2004), we propose that EGCG directs keratinocytes to enter a pathway leading to terminal differentiation and accelerated skin barrier formation.

Our data suggest that the EGCG-induced differentiation requires activation of both p57/KIP2 and caspase 14. The mRNA levels of p57/KIP2 and caspase 14 in NHEK were elevated by EGCG treatment (Fig. 2A). However, notable differences in the times for protein peaks of p57/KIP2 (6 h) versus caspase 14 (20 h) suggested significant p57/KIP2 protein accumulation prior to that of caspase 14. Conversely, acute induction of caspase 14 mRNA by EGCG was observed despite the delayed onset of caspase 14 protein accumulation (Fig. 2). This delayed peak of caspase 14 protein may due to the time required for post-transcriptional processing of pro-caspase 14, which involves proteolytic cleavage and dimerization (Mikolajczyk et al., 2004). In addition, p57/KIP2 was able to induce caspase 14 independently. Retroviral expression of p57/KIP2 in OSC2 cells induced the endogenous transcription of caspase 14 (Fig. 4A). This p57/KIP2 effect was confirmed by pretreatment of S1–S5 cells with TGF-β1 to inhibit p57/KIP2 gene expression, resulting in significant reduction of caspase 14 (Fig. 4B). This result is consistent with the ability of TGF-β1 to inhibit keratinocyte differenti-
ation (Hu et al., 1998) and suggests that p57/KIP2, induced by EGCG in NHEK, may serve as a regulator for caspase 14 expression. In addition, these results imply an integral relationship between p57/KIP2 and induction of keratinocyte differentiation.

In contrast to NHEK, OSC2 cells, which express basal levels of p57/KIP2 and caspase 14 (Fig. 4), undergo caspase 3-dependent apoptosis when exposed to EGCG (Hsu et al., 2003b). However, when exogenous p57/KIP2 was expressed by OSC2 cells (S-clones), cell growth was inhibited up to 100% (S5 cells, Fig. 3A). The S-clones exhibited different degrees of resistance to EGCG-induced cytotoxicity with the exception of S5 cells, which appeared to lose the ability to re-popeulate (Fig. 3B). This failure of repopulation, possibly due to the p57/KIP2 cDNA integration, might cause S5 cells to be less viable in response to EGCG possibly due to induction of differentiation. S1–S4 cells exhibited slower growth rates than OSC2 and G6 cells, suggesting that the expression of exogenous p57/KIP2 resulted in different degrees of growth inhibition, but these cell lines retained the ability to repopulate (Fig. 3B). Although the tumor-specific toxic effects of EGCG were not completely prevented by p57/KIP2 expression in S-clones, a protective role of p57/KIP2 was apparent. This is notable because OSC2 is a metastatic squamous carcinoma line with mutated p53 and silenced p16 (Yoneda et al., 1999). As a member of the KIP/CIP family of proteins involved in cell growth and differentiation (Lee et al., 1995; Deschenes et al., 2001), p57/KIP2 may inhibit mitochondrial-mediated apoptosis by blocking c-Jun N-terminal kinase phosphorylation (Chang et al., 2003), as well as initiation of terminal differentiation by inducing caspase 14. Thus, the protective effect of p57/KIP2 on the S-clones could be closely associated with a cell differentiation pathway. This result is consistent with observations that p57/KIP2 also plays a vital role in both cell differentiation and survival in tissues other than the epidermis (Vattemi et al., 2000; Deschenes et al., 2001). Thus, EGCG-induced p57/KIP2 expression may activate a pathway for cell differentiation.

Recent pathological studies demonstrated that tumor specimens expressed reduced levels of p57/KIP2 protein compared with paired normal tissues, and this reduced expression was often correlated with poor prognosis (Ito et al., 2001, 2002; Nakai et al., 2002), suggesting that the loss of p57/KIP2 expression may contribute to the accelerated growth rate of the more advanced tumors and decreased differentiation. In addition, p57/KIP2 is known to bind to and assist the nuclear translocation of LIM-kinase 1, a downstream effector of the Rho family of GTPases, resulting in the regulation of actin (Yokoo et al., 2003). In this regard, nuclear localization of caspase 14 was observed only in epidermal keratinocytes committed to terminal differentiation, but not in undifferentiated basal cells or psoriatic tissue samples.
This evidence suggests that nuclear translocation of active caspase 14 may guide the cells toward formation of the anucleated stratum corneum. It remains unclear, however, whether p57/KIP2 plays a role in nuclear translocation of caspase 14. Future investigation to better understand the effect of EGCG on epidermal cell modulation will involve the use of animal models such as the “flaky” mouse model with altered keratinization and eventually clinical studies with a focus on treating skin disorders such as psoriasis or improving wound healing.

Acknowledgments

We thank Drs. Fu-Xin Yu and Keeping Xu (Medical College of Georgia) for assisting in the establishment of the p57/KIP2-expressing subclones, Dr. Stephen Elledge (Baylor College of Medicine) for providing p57/KIP2 cDNA, Dr. Mohammad Athar for valuable advice, Haiyan Qin for technical support, and Sharon Barnes for the photographic work.

References


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