Mol. Nutr. Food Res. 2006, *50*, 123 – 129 DOI 10.1002/mnfr.200500125

Review

Opposing action of curcumin and green tea polyphenol in human keratinocytes

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Persistent environmental insult can convert a normal cell into a cancer cell. However, various natural chemopreventive agents called antioxidants can retard this progression. We have recently explored the effects of several chemopreventive agents, including green tea polyphenol and curcumin, on normal human keratinocyte function. Our findings suggest that a bioactive polyphenol from green tea, (−)-epigallocatechin-3-gallate (EGCG), acts to increase involucrin gene expression, suggesting that EGCG treatment enhances normal human keratinocyte differentiation. Mechanistic studies indicate that EGCG alters mitogen-activated protein kinase cascade function to activate involucrin gene transcription *via* a Ras, MEKK1, MEK3, ERK1/2–p38δ cascade that targets AP1 and CAATT enhancer binding protein transcription factors. These findings suggest that EGCG may inhibit disease progression by promoting keratinocyte differentiation. Parallel studies indicate that not all antioxidants produce a similar response. Curcumin, an antioxidant derived from the turmeric, antagonizes the EGCG-dependent response by interfering in this signaling pathway. These studies suggest that different antioxidant may produce antagonistic effects in tissues.

Keywords: Antioxidant / Chemoprevention / Curcumin / EGCG / Green tea

Received: July 26, 2005; revised: October 11, 2005; accepted: October 13, 2005

1 Keratinocyte differentiation

The epidermis is a keratinizing stratified squamous epithelium consisting of layers of keratinocytes, with each layer containing cells at a specific stage in the differentiation process. The keratinocyte stem cells that replenish the epidermis are located in the epidermal basal layer [1, 2]. Differentiating cells cease cell division and manifest an elaborate program of morphological and biochemical change that results in the production of a terminally differentiated cell. Many new proteins are synthesized during this process and these proteins constitute the substrates used to assemble the keratin filament bundles and cornified envelope–structures that are characteristic of fully differentiated keratinocytes [3–6]. The stratification of the epidermal surface is an important strategy devised to protect the organism from environmental hazards including pathogens, environmental carcinogens, and harmful UV light. However, this protection is not perfect, and there are over one million cases of skin cancer diagnosed each year in the United States. Squamous cell carcinoma is the major cancer type which is derived from a prestate called actinic keratosis (AK) [7]. AK cells are mutated in the *p53* gene due to UV light damage [8]. Skin cancer is associated with a loss of cell differentiation; thus, one can presumably reduce the risk of cancer progression by treating with agents that enhance keratinocyte differentiation. In the chemoprevention context, this would require application or consumption of agents that cause mutant keratinocytes to differentiate and cease proliferation prior to tumor formation. As part of an effort to identify such agents, we have studied the role of green tea polyphenols and curcumin as agents that modulate keratinocyte differentiation, using involucrin gene expression as a marker of the differentiation process. Our surprising

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Abbreviations: C/EBP, CAATT enhancer binding protein; **EGCG,** (−)-epigallocatechin-3-gallate; **MAPK,** mitogen-activated protein kinase

results indicate that these agents produce opposing outcomes by differentially regulating a mitogen-activated protein kinase (MAPK) cascade.

2 Involucrin – A marker of keratinocyte differentiation

Involucrin is a structural protein precursor of the keratinocyte-cornified envelope, a protective sheath of covalently crosslinked protein that forms during the final stages of keratinocyte differentiation [9]. Involucrin is incorporated into this structure *via* the action of an enzyme that catalyzes crosslinking, called type I transglutaminase [4, 10]. Involucrin is specifically produced in the suprabasal epidermal layers and is regarded as a marker of keratinocyte differentiation [11, 12]. Moreover, involucrin gene expression is increased by differentiation-inducing agents [13–16]. The mechanisms that regulate involucrin gene expression have been intensively studied [14–22] and so the involucrin gene provides a useful model for understanding the mechanism(s) whereby chemopreventive agents regulate keratinocyte differentiation. A p38δ-ERK1/2 signal transduction cascade, including novel PKC (nPKC) isoforms, Ras, MEKK1, MEK3, and p38δ-ERK1/2 [23], which targets Sp1, CAATT enhancer binding protein (C/EBP), and AP1 transcription factors, regulates involucrin expression [18, 24, 25]. The AP1, C/EBP, and Sp1 transcription factors bind to well-characterized DNA elements within the hINV promoter upstream regulatory region (URR) to activate hINV gene expression [19, 24] (Fig. 1).

Keratinocyte differentiating agents enhance activity in this cascade [16, 18]. Various findings suggest the importance of this cascade in regulating involucrin gene expression. For example, expression of PKCδ, a novel PKC isoform, activates hINV promoter activity [14]. Moreover, activity is increased by constitutively active Ras and inhibited by dominant-negative Ras. These findings indicate that nPKC isoforms and Ras are important for regulation [16]. Similar experimental approaches implicate MEKK1, MEK3, and p38δ [19, 26, 27]. Moreover, the increase in involucrin gene expression that is observed following treatment with keratinocyte differentiating agents is reversed by inhibition at various steps in this cascade. Ultimately, activation of this cascade increases AP1, Sp1, and C/EBP transcription factor levels and increases binding of these factors to the hINV promoter DNA regulatory elements located in the distal (DRR) and proximal regulatory regions (PRR) (Fig. 1).

3 Green tea polyphenol increases hINV promoter activity

(−)-Epigallocatechin-3-gallate (EGCG), a bioactive chemopreventive polyphenol derived from green tea [28–33],

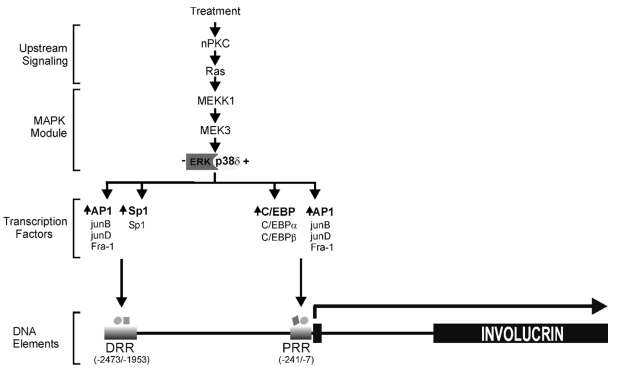

Figure 1. Regulation of involucrin gene expression. Treatment with various keratinocyte differentiating agents (phorbol ester, vitamin D, calcium) activates this MAPK signal transduction pathway to increase involucrin gene expression in keratinocytes [20]. Activation of this cascade results in increased p38δ activity (+) and reduced ERK1/2 activity (−) which leads to an increase in the level (upward small arrows) of the indicated transcription factors. These factors, in turn, interact with specific sites on the hINV promoter to increase gene expression. Involucrin promoter upstream regulatory region includes nucleotides −2473/−1 and two important regions, the DRR (distal regulatory region, nucleotides −2473/−1953) and the PRR (proximal regulatory region, nucleotides −241/−7). Transcriptional regulator elements are indicates as follows: the AP1–5 and AP1–1 sites, represented as spheres; the Sp1 site, represented as a square; and the C/EBP site, represented as a parallelogram. Black rectangles indicate the involucrin exons and the start and direction of transcription is indicated by the arrow [72].

regulates activity in a number of signaling cascades [7, 34–36]. We therefore examined its ability to regulate keratinocyte differentiation and regulate events in the MAPK cascade that lead to activation of involucrin gene expression. Initial studies demonstrated that EGCG treatment increases hINV promoter activity. In these experiments normal human keratinocytes were transfected with a reporter construct, pINV-2473, in which the full-length involucrin promoter (nucleotides –2473/–1) is linked to luciferase. Treatment of the transfected cells with EGCG for 24 h results in an increase in hINV promoter activity (Fig. 2). This increase is nearly as substantial as that observed following treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA), a known inducer of keratinocyte differentiation [23]. Additional studies revealed that EGCG treatment increases expression of endogenous involucrin mRNA and protein [23]. The finding that EGCG increases hINV gene expression is consistent with some previous reports showing that EGCG elicits keratinocyte differentiation, as evidenced by increased expression of differentiation markers and increased transglutaminase activity [37]. Additional promoter studies reveal that an EGCG-response DNA element is located within the proximal regulatory region, (nucleotides −128 to −110) [23] (Fig. 1). Mutation studies

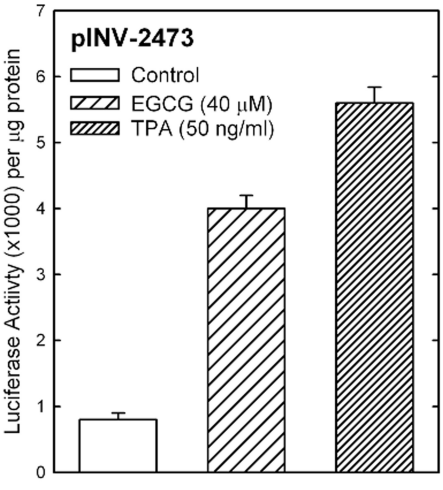

Figure 2. EGCG increases hINV promoter activity. Keratinocytes, growing in KSFM, were transfected 2 μg of pINV-2473, a plasmid that encodes the full-length hINV promoter. Transfected cells were then maintained for 24 h in the absence of treatment (control) or in the presence of 40 μM EGCG or 50 ng/mL TPA. Cells were subsequently harvested for extract preparation and assessment of promoter activity [23].

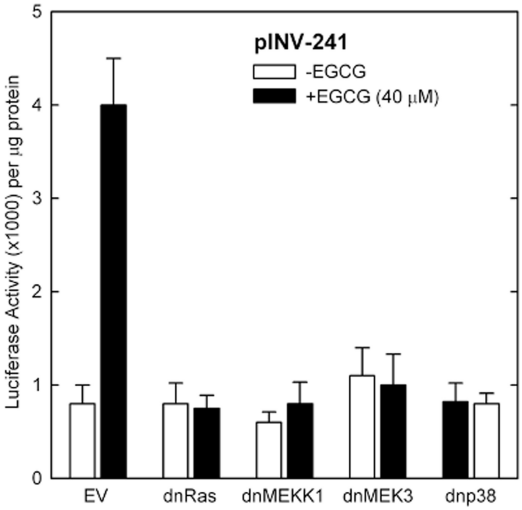

Figure 3. Kinase activities required for EGCG-dependent activation of hINV gene expression. Keratinocytes were transfected 1 μg of pINV-241 in the presence of 1 μg of empty expression vector (EV) or dominant-negative Ras-encoding expression plasmid, dominant-negative MEKK1, dominant-negative MEK3 or dominant-negative p38. Transfected cells were maintained for 24 h in the presence or absence of 40 μM EGCG and assayed for luciferase activity.

reveal that elimination of an AP1 site (AP1-1), present in this region, results in a complete loss of the EGCG-dependent response. Differentiation agents are known to increase AP1 factor level and this increase leads to enhanced involucrin gene expression [18, 25]. Indeed, parallel studies indicate that EGCG acts to increase AP1 factor levels in keratinocytes. Specifically, EGCG treatment increases Fra-1, Fra-2, c-Fos, fos B, c-jun, junB, and junD levels and promotes binding of Fra-1 and junD to the hINV promoter AP1–1 site [23]. These studies demonstrate that EGCG can increase hINV promoter activity, and also indicate that this is accomplished *via* increased binding of AP1 factors to a specific AP1 site within the hINV promoter proximal regulatory region.

4 EGCG–regulation of the p38 MAPK cascade

We next identified the signaling cascade responsible for EGCG-dependent activation of hINV gene expression. Based on our previous studies implicating the mitogen-activated protein kinases in regulation of involucrin gene expression, we focused on members of the cascade shown in Fig. 1. Ras, a small G-protein, is an important regulator of MAPK cascade activity in keratinocytes [16]. In this study we used a truncated form of the involucrin promoter, pINV-241, that encodes the proximal regulatory region. Keratinocytes were transfected with pINV-241 in the presence or absence of expression vectors encoding dominant negative forms of kinases of the MAPK cascade. Treatment with EGCG produces a fourfold increase in hINV promoter activity. However, the presence of dominant-negative forms of Ras, MEKK1, MEK3, or p38 inhibits the EGCG-dependent increase in promoter activity (Fig. 3). These studies suggest that each of these regulatory proteins is engaged in the EGCG-dependent signaling cascade. In contrast, the dominant-negative form of other potential regulators, *e.g.*, Raf-1, MEK6, MEK7, JNK, ERK *etc.*, do not suppress the EGCG regulation [23].

To define the role of individual MAPKs in the EGCG-dependent regulation, we assayed for EGCG-dependent activation of p38(α, β, δ, and γ), ERK1/2, and JNK1/2. JNK1/2 activity is not regulated, and ERK1 and ERK2 are transiently activated following EGCG treatment. In contrast, p38 activity increases rapidly and activity is sustained over 4 h. Additional studies reveal that p38δ is the sole p38 isoform activated in response to EGCG treatment. The net effect at the MAPK level is an increase in p38δ activation relative to ERK1/2 activity. These findings suggest that EGCG activates a Ras, MEKK1, MEK3, p38δ-ERK1/2 cascade. The net effect of EGCG activation of this cascade is a net increase in p38δ activity relative to ERK1/2 activity. This pathway in turn increases AP1, C/EBP, and Sp1 factor level, which bind to their corresponding binding sites on the hINV promoter, to increase promoter activity [23]. Thus, EGCG regulates hINV gene expression in normal human keratinocytes *via* a cascade that is indistinguishable from that utilized by agents that regulate keratinocyte differentiation (Fig. 1).

5 EGCG regulation of AP1 in immortalized/ transformed keratinocytes

In contrast to the EGCG-dependent increase in AP1 factor level observed in normal keratinocytes treated with EGCG, treatment of immortalized or transformed keratinocytes results in a reduction in AP1 factor level [38–42]. Moreover, the EGCG-dependent reduction in AP1 factor expression is associated with inhibition of cell proliferation in murine keratinocytes [39]. Exposure to UVB also increases AP1 factor level in immortalized cells, an increase that is inhibited by EGCG, and EGCG treatment reduces AP1 level in Ha-ras transformed cells [38, 40, 41]. In HaCaT cells, EGCG suppresses the UV light-associated AP1 increase in c-*fos* gene expression [40, 42]. EGCG also inhibits AP1-dependent responses in immortal bronchial epithelial cells [43]. These findings suggest that EGCG may regulate AP1 factor level in an inverse manner in normal *versus* immortalized/transformed surface epithelial cells keratinocytes. This suggests that EGCG treatment may have important advantages as a skin cancer treatment agent. In normal keratinocytes, EGCG treatment may increase AP1-dependent differentiation, and in transformed cells, EGCG may inhibit AP1-associated proliferation – both positive effects with respect to cancer prevention.

6 EGCG increases C/EBP factor-dependent involucrin gene expression

It has previously been shown that C/EBP transcription factors act to increase involucrin gene expression by increasing C/EBP factor level and C/EBP factor binding to the hINV promoter C/EBP site [19, 24] (Fig. 1). In Fig. 4, keratinocytes were transfected with pINV-241 or pINV-241(C/EBPm). The C/EBP site within the hINV promoter, which is contained within the proximal regulatory region, is mutated in pINV-241(C/EBPm). Subsequent treatment with EGCG results in activation of pINV-241. In contrast, EGCG treatment does not cause activation of pINV-241(C/EBPm), suggesting a role for C/EBP transcription factors as mediators of EGCG-dependent activation of hINV gene expression [44]. Consistent with a role for C/EBP factors, expression of the dominant-negative C/EBP factor, GADD153, inhibits EGCG-dependent involucrin promoter activity [44]. Mechanism-oriented studies reveal that C/EBP factor levels increase following EGCG treatment, as does C/EBP factor binding to the hINV promoter C/EBP binding site [44]. This finding suggests that EGCG increases keratinocyte differentiation *via* a least two mechanisms. The first, as described in the previous Sections, is *via* regulation of AP1 factor function. The second, as indicated in this Section, is *via* regulation of C/EBP transcription factor function. As will be described later, curcumin interferes with regulation of C/EBP factor function.

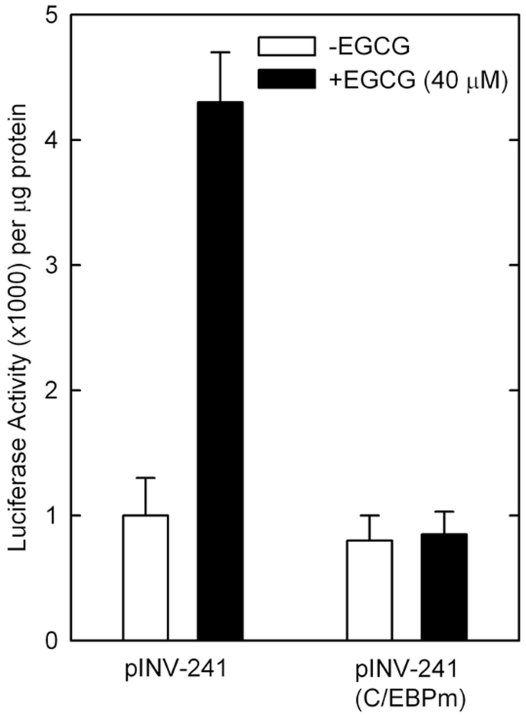

Figure 4. EGCG regulation of hINV promoter activity requires intact C/EBP factor DNA binding site. Human keratinocytes were transfected with pINV-241 or pINV-241(C/EBPm) involucrin promoter reporter constructs. Plasmid pINV-241 encodes an intact hINV promoter. pINV-241(C/EBPm) is identical, except that the C/EBP site is mutated [24]. At 24 h post-transfection, the cells were treated for 24 h in the presence or absence of 40 μM EGCG. Extracts were then prepared for luciferase activity assay.

7 Other EGCG responses in normal human keratinocytes

The above results suggest that EGCG regulates normal keratinocyte function *via* activation of the MAPK kinase cascade. Other published studies also implicate MAPK signaling as an EGCG target. UV light is a major epidermal stress and also results in increased hydrogen peroxide production in cultured normal keratinocytes. This UV exposure is associated with MAPK activation [45], a response that is inhibited by EGCG treatment [45]. EGCG has also been described as producing different effects depending upon the concentration applied. For example, topical treatment with low levels of EGCG enhances epidermal thickness and keratinocyte proliferation [46]. EGCG has also been reported to enhance oxidative stress in transformed keratinocytes [47, 48]. On balance, these studies suggest that EGCG produces differing responses in normal keratinocytes as compared to immortalized keratinocytes and that the concentration of EGCG may be an important determiner of response.

8 Role of curcumin

One can hypothesize that all agents possessing antioxidant activity may produce similar regulatory responses. However, since antioxidants are a structurally diverse group of chemopreventive agents, this outcome cannot be assumed [28, 49]. We therefore used the involucrin promoter response assay to study the role of another antioxidant, curcumin. Curcumin (diferuloylmethane) is a polyphenol derived from the plant *Curcuma longa* [50–52]. We argue that *in vivo* cells are almost certainly simultaneously exposed to multiple antioxidants. However, the effect of simultaneous exposure to multiple antioxidants has not been widely studied. Curcumin is known to inhibit tumor cell proliferation [28, 53, 54] and also to regulate many of the same activities as EGCG [55–63]. We decided to examine the impact of cotreatment of keratinocytes with EGCG and curcumin. Our studies show opposing effects of EGCG and curcumin, and identify at least one mechanism mediating the opposing effects of these agents – curcumin inhibits the EGCG-dependent increase in C/EBP factor expression that is required for involucrin expression.

In these experiments keratinocytes were transfected with pINV-241, and the effects of curcumin and EGCG treatment on hINV promoter activity were monitored. As shown in Fig. 5, curcumin does not influence basal involucrin promoter activity [44]. However, we were surprised to observe that curcumin efficiently inhibits the EGCG-dependent increase in hINV promoter activity in a concentration-dependent manner. Curcumin-dependent regulation of endogenous involucrin gene expression is also observed [44]. Activation of the MAPK cascade at various levels (Fig. 1), using constitutively active kinases, followed by curcumin challenge, reveals that curcumin treatment inhibits constitutively active Ras- and wild-type MEKK1-dependent hINV promoter activation, suggesting that curcumin acts downstream of these effectors. As outlined above, EGCG increases C/EBPα and C/EBPβ levels and binding to the hINV gene promoter response element. To gain further insight regarding the mechanism of the curcumin-dependent inhibition, we monitored the ability of curcumin to inhibit C/EBPα-dependent hINV promoter activity. These studies reveal that curcumin causes a reduction in C/EBPα and β levels, a decrease that correlates with reduced C/EBP factor binding to the hINV promoter C/EBP site and reduced involucrin transcription [44].

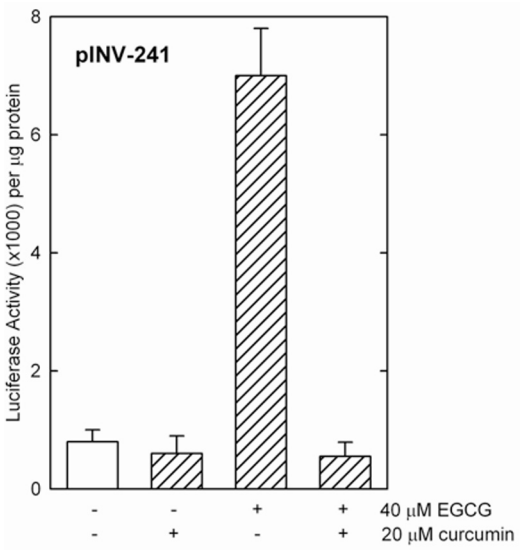

Figure 5. Curcumin opposes the EGCG-associated increase in promoter activity. Keratinocytes were grown in KSFM until 70% confluent and then transfected with the pINV-241 luciferase reporter construct [44]. At 24 h after transfection, the cells were treated for 24 h with or without 40 μM EGCG and/or 20 μM curcumin prior to harvest and assay for luciferase activity.

9 Curcumin opposes the EGCG effects on involucrin gene expression *via* regulation of C/EBP factor level – Role of the proteasome

Many proteins, including C/EBP factors, are degraded by proteasome-dependent mechanisms [64, 65], and proteasome inhibitor treatment increases C/EBP factor level in a variety of cell types [66–68]. Curcumin has also been reported to regulate proteasome function [69]. Mukhopadhyay *et al.* [70], studying prostate cancer cells, showed that a curcumin-dependent decrease in cyclin D1 level is inhibited by a proteasome inhibition. Indeed, our recent report indicates that curcumin inhibits the EGCG-dependent activation of involucrin promoter activity by inhibiting the EGCG-promoted increase in C/EBP factor level. An especially intriguing finding is that the curcumin-associated reduction in C/EBP factor levels is reversed by treatment with MG132, an inhibitor of proteasome function [44]. This finding suggests that curcumin treatment targets C/EBP transcription factors for proteasome-associated degradation. Thus, while EGCG treatment increases C/EBP factor levels, treatment with curcumin antagonizes this effect by promoting proteasome-dependent C/EBP factor degradation.

10 Conclusions: Relevance to cancer biology and chemoprevention

In cultured normal human keratinocytes EGCG increases involucrin expression *via* activation of a Ras, MEKK1, MEK3, p38δ-ERK pathway [16, 71]. Our studies show that EGCG treatment produces a shift in the balance of p38δ to ERK1/2 activity in favor of p38δ [23]. The EGCG-depen-

dent shift in the level of p38δ-ERK activity in favor of p38δ in turn causes increased C/EBP and AP1 factor expression, increased binding of these factors to the hINV promoter, and increased hINV gene expression [23]. Curcumin, in contrast, inhibits the EGCG-dependent increase in promoter activity. Mechanistic studies suggest that this opposition is due to the fact that curcumin inhibits the EGCG-dependent increase in C/EBP factor levels, and reduces C/EBP binding to the hINV promoter C/EBP site. Additional studies reveal that curcumin may act to target C/EBP factors for degradation *via* a proteasome-associated mechanism [44]. Since C/EBP factor action is required for hINV promoter activity, the reduction in C/EBP factor level reduces involucrin gene expression. Curcumin may also influence the stability of other hINV gene regulatory factors such as Sp1 and AP1 factors, a possibility that is presently being explored. We suggest, based on these studies, that the potential opposing biological roles of antioxidants must be considered when designing chemopreventive therapies.

The present studies have some potentially important implications for cancer chemoprevention. First, the study indicates that chemopreventive agents can promote keratinocyte differentiation. This is a potentially important finding, since keratinocytes are primary carcinogen targets, and causing them to differentiate may be a mechanism to remove mutagenized cells from the epidermis. Second, two chemopreventive antioxidants may cause different, and perhaps opposing effects, in keratinocytes. This finding suggests that not all antioxidants are created equal. This is a finding that might be anticipated, based on the structural differences among the various families of antioxidants, but has not been previously emphasized. This finding not only suggests that some antioxidants may neutralize that action of other antioxidants but also implies that synergistic actions may be observed. Third, chemopreventive agents can produce different responses in normal *versus* immortalized/transformed keratinocytes. Thus, in normal keratinocytes EGCG increases AP1 factor levels, while in immortalized/transformed keratinocytes EGCG treatment reduces AP1 factor expression. This suggests that the activity and mechanism of chemopreventive agent action can change during disease progression and implies that different chemopreventive agents may have different efficacy depending upon the disease stage. Fourth, chemopreventive agents may be simultaneously antagonizing and synergistic. For example, treatment of keratinocytes with EGCG increases AP1 and C/EBP factor levels and involucrin gene expression (differentiation) and curcumin inhibits this action; however, both agents suppress keratinocyte proliferation. In fact, combined treatment with EGCG and curcumin results in more efficient suppression of cell proliferation than treatment with each agent alone (Balasubramanian and Eckert, unpublished). Thus, in spite of the fact that EGCG and curcumin have opposing action on differentiation, they may still be effective when used together because of the shared property of growth suppression. Based on our findings, we propose that both EGCG and curcumin will cause cancer chemoprevention, EGCG by promoting keratinocyte differentiation, and EGCG and curcumin by suppressing keratinocyte proliferation and survival. Thus, we speculate that the combined treatment with both agents will be more effective than that of each single agent, largely because both share a common ability to inhibit keratinocyte proliferation. The ultimate lesson to be derived from these studies is that not all antioxidant chemopreventive agents are created equal and that one may expect surprising types of regulation when two chemopreventive agents are used simultaneously.

This work used the facilities of the Skin Diseases Research Center of Northeast Ohio (AR39750) and was supported by grants from the National Institute of Health (RLE). Dr. Balasubramanian received a World Green Tea Association O-CHA(Tea) Pioneer Research Grant, and Dr. Efimova was supported by a Dermatology Foundation Career Development Award.

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