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Antioxidants regulate normal human keratinocyte differentiation

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Abstract

Cancer begins with a normal cell that, due to persistent environmental insult, is transformed, via a series of progressively more insidious steps, into a cancer cell. A major goal of chemopreventive therapy is to alter the normal cell response to the environmental agent with the goal of inhibiting disease progression. (−)-Epigallocatechin-3-gallate (EGCG) is an important bioactive green tea antioxidant that possesses remarkable cancer chemopreventive properties. We have recently explored the hypothesis that EGCG prevents cancer by promoting keratinocyte differentiation. Based on our findings, we argue that EGCG acts to enhance the differentiation of normal keratinocytes. This is a potentially important finding, as it represents a novel mechanism of disease inhibition by EGCG – cancer preventive ''differentiation therapy''. However, not all antioxidant chemopreventive agents work by this mechanism. Curcumin, for example, inhibits the differentiation-promoting activity of EGCG. This report discusses the mechanism of EGCG and curcumin action in regulating expression of involucrin, a marker of keratinocyte differentiation.

Keywords: EGCG; Green tea; Chemoprevention; Curcumin; Antioxidant; Skin cancer

1. Regulation of keratinocyte differentiation

The keratinocyte is the major cell type of the epidermis and the cell type responsible for construction of the epidermal surface. The basal epidermal layer contains the keratinocyte stem cell population that provides a continuous supply of cells that replenish the epidermis [1,2]. These cells undergo a transient amplification to produce daughter cells that then leave the basal layer to begin the journey to the epidermal surface – the process of differentiation. Two important events occur during keratinocyte differentiation: first, the keratinocyte stops proliferating, and second, the cell undergoes a programmed set of morphological and biochemical changes that result in the production of a terminally differentiated cell, the corneocyte. Biochemical changes include the synthesis of new keratinocyte proteins including specific cytokeratins and cornified envelope precursors [3,4]. These differentiation products are then used to construct the distinctive features of the differentiated keratinocyte – the keratin filament bundlers and the cornified envelope. Taken together, the composite of billions of terminal keratinocytes (cornified envelopes) form the epidermal surface that protects against pathogens, environmental carcinogens and ultraviolet light.

The surface of the epidermis is subject to a wide variety of environmental insults that can lead to the development of squamous cell carcinoma. There are over one million cases of skin cancer diagnosed each year in the US. Most of these are squamous cell carcinomas that develop following chronic ultraviolet light (UV) exposure. Actinic keratosis (AK) has been identified as a precursor for squamous cell carcinoma [5]. Actinic keratosis is characterized by the presence of p53 mutations due to ultraviolet light damage [6]. A possible strategy for treating these types of diseases at the early stage is causing the cells to undergo terminal differentiation with elimination from the epidermal

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surface. Based on our preliminary studies showing that EGCG treatment increases involucrin gene expression, we hypothesized that antioxidants may enhance keratinocyte differentiation. In the present report we describe studies suggesting that green tea polyphenol (EGCG) enhances keratinocyte differentiation via regulation of a p38δ-ERK cascade leading to activation of hINV gene expression.

2. Regulation of involucrin expression

Involucrin is an important marker of keratinocyte differentiation [7,8]. Involucrin is produced in the suprabasal layers (late spinous/granular) and is a precursor of the cornified envelope. The cornified envelope is a protective protein sheath that forms beneath the plasma membrane during the final stages of keratinocyte differentiation. Involucrin incorporation into the envelope is catalyzed via formation of covalent isopeptide bonds via the action of type I transglutaminase [9,10]. Involucrin gene expression, in cultured cells, is controlled by a variety of keratinocyte differentiation regulating agents, including calcium, phorbol ester and others [11–14]. Because of this differentiation-dependent pattern of expression, and because the mechanism(s) of regulation are well studied [12–27], the involucrin gene provides an ideal model for understanding the mechanism(s) whereby chemopreventive agents regulate normal keratinocyte differentiation. Involucrin gene expression is regulated via a p38δ-ERK1/2 signal transduction cascade. This cascade includes novel PKC (nPKC) isoforms, Ras, MEKK1, MEK3, and p38δ-ERK1/2 [28], and targets Sp1, C/EBP and AP1 transcription factors [16,20,29]. These transcription factors then bind to specific DNA element within the hINV promoter upstream regulatory region (URR; nucleotides −2473/−1) (Fig. 1) to activate hINV gene expression [20,24].

Treatment with keratinocyte-differentiating agents results in increased activity in the p38δ-ERK cascade [14,16]. Moreover, this activity is directly linked to increased hINV promoter activity. This regulatory pathway is presented schematically in Fig. 1. To understand this regulation, we examined the effect of various upstream kinases on activation of hINV promoter activity. Transfection of involucrin promoter reporter plasmid with plasmid encoding PKCδ, a novel PKC isoform, results in activation of hINV promoter activity [12], suggesting a role for PKCδ (and other novel PKC isoforms) in regulating hINV gene expression. In addition, this cascade is activated by constitutively active Ras and is inhibited by dominant-negative Ras, indicating that Ras function is requisite for this regulation [14]. Transfection of cells with MEKK1 expression plasmid results in increased hINV promoter activity and this activation is inhibited by dominant-negative MEK3 and dominant-negative p38, suggesting that

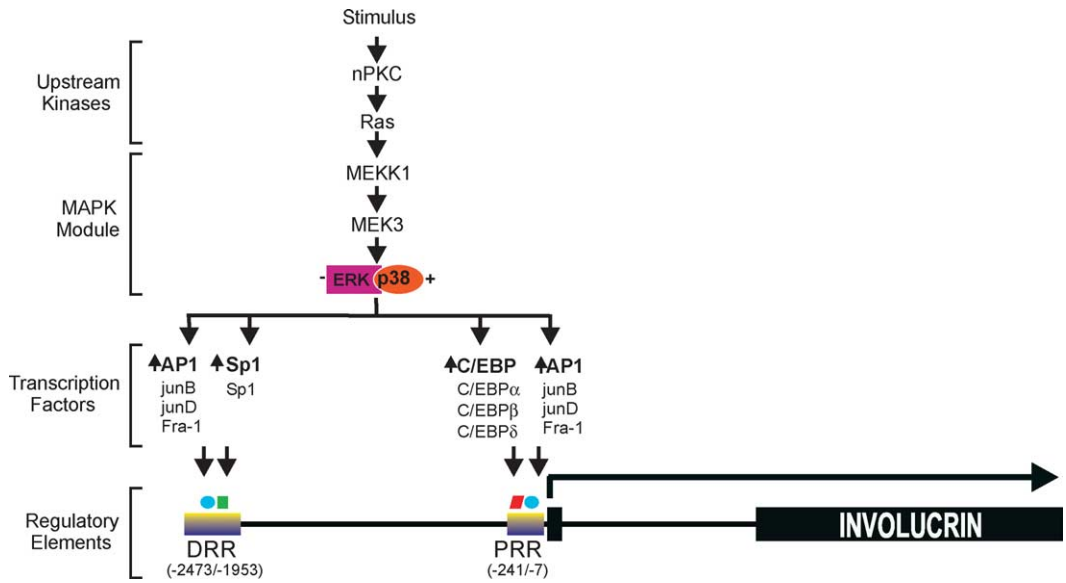

Fig. 1. MAPK regulation of involucrin gene expression. The differentiating stimulus (phorbol ester, vitamin D, calcium) activates the indicated signal transduction pathway which ultimately alters the activity of the p38δ-ERK1/2 mitogen-activated protein kinases which are part of a MAPK signaling complex that exists in resting and stimulated keratinocytes [25]. This results in increased p38δ activity (+) and reduced ERK1/2 activity (−) without changing the level of either kinase. This shift in activity results in an increase in the level (upward small arrows) of the indicated transcription factors which interact with the appropriate sites within the hINV promoter to increase hINV promoter activity. The upstream regulatory region of the involucrin promoter includes nucleotides −2473/−1 and includes two important regions, the distal regulatory region (DRR; nucleotides −2473/−1953) and the proximal regulatory region (PRR; nucleotides −241/−7). Important transcriptional regulatory elements are indicated. These include the AP1-5 and AP1-1 sites, represented as circles, the Sp1 site, represented as a square, and the C/EBP site, represented as a parallelogram. The two involucrin exons are indicated by the black rectangles and the start and direction of transcription is indicated by the arrow [86]. Various keratinocyte-differentiating agents regulate hINV gene expression via this pathway [14]. As described in this report, EGCG also appears to regulate hINV expression via this pathway [28].

MEKK1 is upstream of MEK3 and p38 MAPK [22–24]. Moreover, calcium and phorbol ester-dependent activation of hINV gene expression is inhibited in the presence of dominant-negative forms of these kinases, further suggesting that they are part of the regulatory cascade. Activation of this cascade results in increased levels of AP1, Sp1 and C/EBP transcription factors and increased binding of these factors to the hINV promoter DNA regulatory elements located in the distal (DRR) and proximal regulatory regions (PRR). An important issue is how EGCG may regulate these events.

3. EGCG treatment increases hINV promoter activity

(−)-Epigallocatechin-3-gallate (EGCG) is an important bioactive polyphenol, isolated from green tea, that possesses remarkable anticancer and chemopreventive activity [30–36]. It has a range of bioactivities and regulates activity in several signaling cascades [5,37–41]. Keratinocytes, like other cell types, have systems designed to handle redox-related stress. These systems buffer the intracellular environment and protect against the negative effects of reactive oxygen species. However, excessive exposure to reactive oxygen species can cause damage to cellular components and cause cancer [42]. In these circumstances, application of natural or synthetic antioxidants, such as EGCG, an important natural antioxidant, may provide protection [43]. In the present study, we examine the role of EGCG in regulating normal human epidermal keratinocyte function.

Our initial studies with EGCG showed that treatment of normal keratinocytes for 24 h with 20 μg/ml EGCG results in an activation of hINV promoter activity that is equivalent to that observed following treatment with 50 ng/ml TPA [28]. Expression of the endogenous gene is also increased by treatment with EGCG, indicating that the response is physiologically relevant, and suggesting that EGCG increases keratinocyte differentiation. Other investigators have also suggested a role for EGCG in regulating keratinocyte differentiation. Hsu et al. showed that EGCG elicits cell differentiation responses, as evidenced by increased keratin K1 and filaggrin expression, and increased transglutaminase activity [44].

Involucrin promoter deletion studies reveal that the EGCG-response DNA element is located within the promoter segment spanning nucleotides −128 to −110. This region of the hINV promoter is located within the proximal regulatory region (Fig. 1). This region encodes a functional ets factor binding site (EBS-2), and an activator protein 1 (AP1-1) transcription factor binding site [28]. Mutation studies reveal that the EBS-2 site is not required for EGCG regulation. However, mutation of the AP1-1 site results in a complete loss of EGCG-dependent regulation. Previous studies show that differentiation agents increase AP1 factor level and this increase is associated with increased gene expression [16,29]. To determine whether EGCG increases hINV gene expression by a similar mechanism, we assessed the effect of EGCG on AP1 factor level. These studies demonstrated that EGCG increases Fra-1, Fra-2, c-Fos, fos B, c-jun, junB and junD level [28]. The increased level of these factors is associated with increased complex formation at the hINV promoter AP1-1 site. Gel mobility supershift studies reveal that Fra-1 and junD bind to the AP1-1 site in an EGCG-dependent manner [28].

4. EGCG increases activity in the p38 MAPK cascade

Initial experiments indicate that EGCG-dependent activation of hINV promoter activity is inhibited by genistein treatment. Genistein is an efficient inhibitor of tyrosine kinases, including the dual specific MAPK kinases (MEK) [28]. We then initiated studies to determine whether other proteins involved in involucrin gene regulation are also activated. We began with Ras, a small G-protein, as Ras is an important regulator of MAPK cascade activity in keratinocytes [14]. These studies reveal that dominant-negative Ras suppresses the EGCG-dependent increase in hINV promoter activity. In addition, a dominant-negative form of MEKK1 also inhibits promoter activation, suggesting a positive regulatory role for MEKK1. In contrast, dominant-negative Raf-1 (dnRaf-1) does not alter the response [28].

MEKK1 kinase is known to activate several downstream MAPK pathways [45]. MEK4, MEK7 and MEK3 are direct downstream targets of MEKK1 [14,22]. Dominant-negative mutants of these kinases were used to identify which of these kinases convey the MEKK1-dependent signal in the EGCG-response cascade. Among these kinases, only dominant-negative MEK3 inhibits the EGCG-dependent activation of hINV promoter activity, suggesting that MEK3 is the downstream target of MEKK1. To assess the ability of individual MAPKs to function as mediators downstream of MEK3, we measured the ability of various dominant-negative MAPKs to inhibit the EGCG-related response. Although some effects are observed with dominant-negative ERK1/2 and dominant-negative JNK, the most striking response is evoked by dominant-negative p38, which completely inhibits the response to EGCG. Based on these results, we suggest that EGCG regulates hINV gene expression via the same pathway used by known differentiation regulatory agents, as shown in Fig. 1.

To further examine the role of individual MAPKs in the EGCG-dependent regulation, we assayed for EGCG-dependent activation of p38α, -β, -δ and -γ, ERK1/2, and JNK1/2. ERK1 and ERK2 are transiently activated at 20 min following EGCG treatment. In contrast, p38 activity increases by 15 min and remains elevated at 4 h. JNK activity is not regulated. To determine which p38 isoform is activated, keratinocytes expressing

FLAG-tagged p38α, -β, -δ or -γ were treated with EGCG for 24 h and the activity of each isoform was monitored. This study reveals that p38δ is the only p38 isoform activated by EGCG treatment. Thus, EGCG activates a Ras, MEKK1, MEK3, p38δ-ERK1/2 cascade that increases AP1, C/EBP and Sp1 factor level to increase hINV gene expression [28]. Moreover, this cascade is essentially identical to that which mediates regulation by agents that are known to increase keratinocyte differentiation. In addition to increasing involucrin expression, EGCG also suppresses keratinocyte proliferation and enhances cornified envelope formation [28], two hallmarks of the keratinocyte differentiation process [7].

The above studies indicate that EGCG increases AP1 factor level in normal human keratinocytes. This regulation differs substantially from that observed in immortalized and/or transformed keratinocytes. In immortalized and transformed keratinocytes, AP1 transcription factor level is increased by carcinogenic stimuli that increase cell proliferation. EGCG inhibits this increase [46–50]. In mouse keratinocytes, cell transformation is inhibited by EGCG and this is associated with reduced AP1 factor expression and DNA interaction [47]. EGCG also inhibits the increase in AP1 level observed following UVB exposure [46,49]. In HaCaT cells, EGCG inhibits the UVB-dependent increase in AP1, the transcriptional activation of c-fos gene and accumulation of c-Fos protein [48,50]. EGCG also suppresses AP1-dependent events in immortalized bronchial epithelial cells [51].

5. EGCG increases C/EBP factor-dependent involucrin promoter activity

As noted above, in addition to AP1 factors, C/EBP factors are also important regulators of involucrin gene expression [20,24]. Treating keratinocytes with various differentiation regulators results in increased expression of selected C/EBP factors and increased binding of these factors to a C/EBP site within the hINV promoter (see Fig. 1). Moreover, mutation of the hINV promoter C/EBP site results in a loss of this regulation [20]. Initial studies indicate that EGCG treatment increases hINV promoter activity and that mutation of the C/EBP site within the hINV promoter proximal regulatory region, see Fig. 1, results in a loss of EGCG-dependent activation [52]. In addition, expression of the dominant-negative C/EBP factor, GADD153, inhibited the EGCG-dependent increase in promoter activity. These results suggest that EGCG may increase hINV promoter activity via activation of C/EBP factor function. To understand the mechanism, we monitored C/EBP factor levels following treatment of keratinocytes with EGCG. These studies showed that EGCG treatment increases the level of C/EBPα and -β and also increases C/EBP transcription factor complex formation at the hINV promoter C/EBP site [52].

6. Other MAPK-dependent responses to EGCG in normal keratinocytes

The effect of EGCG on MAPK signaling in normal keratinocytes has also been monitored in other contexts. As mentioned above, ultraviolet light is a major stress to the surface of the epidermis that results in the production of an intracellular oxidizing environment. Thus, UVB exposure results in increased hydrogen peroxide production in culture normal keratinocytes which leads to MAPK activation [53]. EGCG treatment inhibits the UVB/peroxide-dependent increase in ERK1/2, p38 and JNK activity [53]. In addition, a recent report suggests that topical treatment with low levels of EGCG enhances epidermal thickness and keratinocyte proliferation [54]. Other studies suggest that EGCG is exclusively cytotoxic to transformed keratinocytes as it preferentially enhances oxidative stress in these cells [55,56]. Taken together, these studies suggest that the response of normal human epidermal keratinocytes to EGCG treatment is concentration and context dependent. Additional studies will be necessary to identify the underlying mechanisms that mediate these differing responses.

7. Role of curcumin

Antioxidants are a diverse group of agents that are potential disease-preventive agents [30,57,58]. One can speculate that any compound possessing antioxidant properties could increase involucrin gene expression in a manner similar to that observed for EGCG. To investigate this possibility, we examined the effects of another antioxidant, curcumin, on involucrin gene expression. Curcumin (diferuloylmethane), commonly called turmeric, is a polyphenol derived from the plant *Curcuma longa* [59–61]. We examined the ability of curcumin to regulate involucrin gene expression. In contrast to EGCG, curcumin did not alter basal involucrin gene expression [52]. We next treated cells with EGCG in the presence of increasing levels of curcumin. Curcumin produced a concentration-dependent inhibition of EGCG-dependent hINV promoter activity. To identify the mechanism whereby curcumin antagonizes the EGCG-dependent activation of involucrin gene expression, we activated the MAPK cascade at various levels using constitutively active kinases and then monitored the ability of curcumin to inhibit hINV promoter activity. Curcumin treatment inhibits constitutively active Ras- and wild-type MEKK1-dependent hINV promoter activity, 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 insights 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 that correlates

with reduced C/EBP factor binding to DNA and involucrin transcription [52]. Studies in other systems indicate that C/EBP is degraded via a proteasome-dependent mechanism. Indeed, treatment with proteasome inhibitor reverses the curcumin-dependent reduction in C/EBPα and -β level, suggesting that curcumin targets C/EBP factors for proteasome-dependent degradation.

8. Curcumin opposes the action of EGCG in normal epidermal keratinocytes

An important consideration in therapeutics is the fact that cells are simultaneously exposed to multiple antioxidants. The effect of simultaneous treatment with multiple antioxidants has not been widely studied. In the present studies, we examined how two different antioxidants, EGCG and curcumin, regulate keratinocyte differentiation [62,63]. Like EGCG, curcumin inhibits tumor cell proliferation [30,64,65] and regulates many of the same activities as EGCG [66–74], including skin carcinogenesis and skin tumor cell apoptosis [64,75–77]. Both EGCG and curcumin inhibit oral epithelial cell growth, and combined treatment results in enhanced growth inhibition [78]. In contrast, our studies show opposing effects of these agents. We identify at least one mechanism responsible for the opposing actions of EGCG and curcumin. EGCG treatment increases the level of two immediate upstream regulators of involucrin expression, C/EBPα and -δ. Curcumin inhibits this activation.

Many proteins, including C/EBP factors, are degraded by proteasome-dependent mechanisms [79,80]. C/EBPα has a half-life of 1 h in BALB/MK2 cells, but proteasome inhibitors increases the half-life and C/EBPα level increases five-fold [81]. Proteasome inhibitor treatment of Caco-2 cells also increases C/EBPβ and C/EBPδ levels [82]. Moreover, leucine zipper-dependent dimer formation, between two C/EBP monomers, protects C/EBP factors from degradation [83]. Some experiments suggest that curcumin regulates proteasome function. In mesangial cells, for example, curcumin inhibits proteasome inhibitor-dependent increase in monocyte chemoattractant protein expression [84]. In addition, Aggarwal and coworkers showed that a curcumin-dependent reduction in cyclin D1 level in prostate and breast cancer cells is inhibited by proteasome inhibitor, lactacystin [85]. These studies, in conjunction with our study, suggest that curcumin may target proteins for proteasome-dependent degradation.

9. Summary

Our studies suggest that EGCG increases involucrin expression via activation of a Ras, MEKK1, MEK3, p38δ-ERK pathway. We hypothesize that activation of this pathway results in a shift in the level of p38δ/ERK1/2 activity in favor of p38δ. This shift results in an increase in C/EBP and AP1 transcription factor levels. The increased level of these factors results in increased complex formation at C/EBP and AP1 factor binding sites in the hINV promoter which results in increased hINV gene transcription [28]. Curcumin, in contrast, does not regulate basal involucrin gene expression; however it does inhibit the EGCG-dependent increase in promoter activity. Mechanistic studies reveal that curcumin inhibits the EGCG-dependent increase in C/EBP factor level via a mechanism that appears to involve increased proteasome-dependent C/EBP factor degradation. Since C/EBP factor action is required for hINV promoter activity, the reduction in C/EBP factor level reduces involucrin gene expression. We anticipate that curcumin may also influence the stability of other transcription factors that regulate hINV gene expression, including Sp1 and AP1 factors. Additional studies will be necessary to assess these possibilities. These findings suggest that the potential opposing biological roles of antioxidants should be considered when designing chemopreventive therapy.

Acknowledgments

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