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KERATINOCYTE PROLIFERATION, DIFFERENTIATION, AND APOPTOSIS - DIFFERENTIAL MECHANISMS OF REGULATION BY CURCUMIN, EGCG AND APIGENIN

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Abstract

We have proposed that it is important to examine the impact of chemopreventive agents on the function of normal human epidermal keratinocytes, since these cells comprise the barrier that protects the body from a range of environmental insults. In this context, it is widely appreciated that cancer may be retarded by consumption or topical application of naturally-occurring food-derived chemopreventive agents. Our studies show that (−)-epigallocatechin-3-gallate (EGCG), a green tea-derived polyphenol, acts to enhance the differentiation of normal human keratinocytes as evidenced by its ability to increase involucrin (hINV), transglutaminase type 1 (TG1) and caspase-14 gene expression. EGCG also stimulates keratinocyte morphological differentiation. These actions of EGCG are mediated via activation of a nPKC, Ras, MEKK1, MEK3, p38δ-ERK1/2 signaling cascade which leads to increased activator protein 1 (AP1) and CAATT enhancer binding protein (C/EBP) transcription factor expression, increased binding of these factors to DNA, and increased gene transcription. In contrast, apigenin, a dietary flavonoid derived from plants and vegetables, and curcumin, an agent derived from turmeric, inhibit differentiation by suppressing MAPK signal transduction and reducing API transcription factor level. Curcumin also acts to enhance apoptosis, although EGCG and apigenin do not stimulate apoptosis. In addition, all of these agents inhibit keratinocyte proliferation. These findings indicate that each of these diet-derived chemopreventive agents has a profound impact on normal human keratinocyte function and that they operate via distinct and sometimes opposing mechanisms. However, all are expected to act as chemopreventive agents.

Keywords

EGCG; apigenin; curcumin; TPA; epidermis; involucrin; keratinocyte differentiation; apoptosis; chemoprevention; turmeric

Keratinocyte differentiation and human involucrin gene expression

The keratinocyte is the major cell type of the multilayered stratified squamous epithelium (the epidermis) that covers the body surface. To establish epidermal structure, keratinocytes in the basal layer undergo periodic cell division which gives rise to daughter cells that differentiate

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to produce the suprabasal epidermal layers (Nemes and Steinert, 1999). The epidermis provides protection against cancer-promoting agents present in the environment. UV light exposure, for example, is a major cause of human skin cancer and so identifying agents that prevent UV-dependent cancer is an important strategy (Lu et al., 2002). Consumption of dietary agents that reduce keratinocyte proliferation and enhance the conversion of pre-malignant cells to differentiated cells is expected to reduce cancer development. Thus, a major goal is to identify such agents and to understand their mechanism of action. We have focused on identifying agents that regulate the differentiation of normal human keratinocytes. This is an important effort, since the normal keratinocyte is the cell that provides the interface between the body and the environment and, as such, is exposed to a wide range of potentially carcinogenic agents.

Involucrin is an AP1 transcription factor-regulated marker of keratinocyte differentiation that is expressed in the suprabasal (late spinous/granular) layers of the human epidermis. Keratinocyte differentiating agents, including calcium and phorbol ester, activate involucrin gene expression (Eckert et al., 2004; Efimova et al., 2002; Efimova et al., 2003) via a signaling cascade that includes the novel PKC isoforms (nPKC), Ras, MEKK1, and MEK3 (Agarwal et al., 1999; Welter et al., 1995; Efimova and Eckert, 2000; Efimova et al., 1998). Activation of this cascade leads to an increase in p38δ and a decrease in ERK1/2 activity leading to an increase in the levels of AP1, Sp1 and CAATT enhancer binding protein (C/EBP) expression, increased binding of these factors to the hINV promoter, and increased hINV gene expression (Banks et al., 1998; Welter et al., 1995; Crish et al., 2002). Evidence for this cascade is provided by studies using pharmacological agents, constitutively-active and dominant-negative kinases, and kinase assays (Efimova and Eckert, 2000; Efimova et al., 1998; Efimova et al., 2002; Efimova et al., 2003). As a model to study the impact of dietary agents on keratinocyte function, we have studied the impact of a range of antioxidants on expression of involucrin (Crish et al., 1993; Crish et al., 1998; Crish et al., 2002; Eckert et al., 2004). We have also examined the impact of treatment with chemopreventive agents on keratinocyte proliferation and apoptosis. It is anticipated that an effective chemopreventive agent may enhance keratinocyte differentiation, suppress keratinocyte proliferation, enhance keratinocyte apoptosis, or produce a combination of these changes. In this brief review, we compare the regulation by (−)-epigallocatechin-3-gallate (EGCG), apigenin and curcumin on these processes.

Chemopreventive agents

Green tea polyphenols

Green tea has been reported to be effective against a number of cancers including skin, oral cavity, esophagus, stomach, lung, liver, prostate, bladder, cervix, colon, and small intestine (Stoner and Mukhtar, 1995). Green tea contains several polyphenols; however, EGCG is the most abundant polyphenol present in green tea and is believed to be responsible for most of the cancer chemopreventive properties. EGCG has been reported to induce apoptosis and promoter cell growth arrest by altering expression of cell cycle regulatory proteins, altering Bax/Bcl2 function, activating killer caspases, and suppressing nuclear factor kappa B function (Gupta et al., 2004; Khan et al., 2006). EGCG is an effective cancer preventive agent in mouse skin carcinogenesis and inhibits skin cancer cell proliferation *in vitro* (Katiyar et al., 2001; Katiyar et al., 1997). It prevents TPA and epidermal growth factor (EGF)-induced transformation of mouse JB6 epidermal cells via suppression of JNK phosphorylation and AP1 activation (Dong et al., 1997). It also inhibits UV-light dependent activation of c-fos gene and protein expression (Chen et al., 1999).

Apigenin

Apigenin is a dietary flavonoid found at high levels in parsley, thyme, and peppermint, and also in some herbs. Because of its potential antioxidant, anti-inflammatory, and anti-tumor

properties, apigenin is a candidate cancer chemopreventive agent (Birt et al., 1997; Birt et al., 1996; Lepley et al., 1996; Ross and Kasum, 2002). Apigenin treatment inhibits cell proliferation in cancer cell types (Sarkar and Li, 2004). Apigenin treatment reduces the number and the size of skin tumors that develop in response to chemical carcinogen or UVB exposure via a mechanism that involves inhibition of ornithine decarboxylase activity (Wei et al., 1990). Apigenin also inhibits the TPA-dependent increase in c-jun and c-fos gene expression and tumor promotion in mouse skin (Huang et al., 1997c), and suppresses TPA-mediated COX-2 expression by blocking Akt signal transduction and arachidonic acid release in HaCaT cells (Van Dross et al., 2005).

Curcumin

Curcumin is an important polyphenol derived from the rhizome *Curcuma longa L*. Curcumin has anti-inflammatory, antioxidant, anticarcinogenic, antiviral, and antiinfectious activity (Shishodia et al., 2005). These functions are attributable to curcumin's regulation of the function of various transcription factors including NF-kB, AP1, EGr-1 and C/EBP, and the suppression of genes encoding TNF, COX-2, chemokines and cell adhesion proteins. Curcumin suppress the proliferation of cancer cells via its effects on cell cycle, apoptosis and differentiation (Huang et al., 1988a; Huang et al., 1997b; Huang et al., 1997a; Huang et al., 1988b). Curcumin treatment has been shown to suppress skin tumor development in 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate treated mice (Huang et al., 1997a; Limtrakul et al., 2001; Singletary et al., 1998). Curcumin also attenuates the TPA-dependent increase in skin inflammation, hyperplasia, DNA synthesis, c-fos and c-jun protein expression, and ornithine decarboxylase (ODC) activity (Huang et al., 1997b).

Chemopreventive agents differentially regulate hINV gene expression

When we initiated these studies, the role of chemopreventive agents in regulating normal human epidermal keratinocyte function had not been extensively studied, as most investigations focus on transformed keratinocytes. However, we have argued that it is important to examine the impact of chemopreventive agent treatment on the function of normal epidermal keratinocytes, since these cells comprise the interface between the body and the environment. As such, these cells are exposed to a wide range of mutagenic environmental challenges and so it makes sense to assess whether chemopreventive agents regulate their function. For example, an agent that enhances the differentiation of normal or pre-malignant human keratinocytes, and thereby removes the cell from the proliferative pool, may provide a substantial anti-cancer advantage.

We initiated our studies to determine whether these agents regulate keratinocyte differentiation by assessing the impact of treatment on transcription of the involucrin (hINV) gene (Eckert et al., 2004). Keratinocytes were transfected with an involucrin promoter-luciferase reporter plasmid, pINV-241, in which the proximal hINV promoter is linked to luciferase (Balasubramanian et al., 2002; Balasubramanian and Eckert, 2004). Our studies showed that treatment with EGCG for 24 h activates the human involucrin promoter reporter plasmid, pINV-241. Immunoblot and RT-PCR analyses show that EGCG treatment also increases endogenous hINV protein and mRNA levels. We next investigated the effects of apigenin and curcumin on hINV expression. Hsu and coworkers recently reported that EGCG causes similar differentiation-dependent event, including increased expression of p57/KIP2, keratin 1, and filaggrin, and increased transglutaminase activity in normal human keratinocytes (Hsu et al., 2005). Our investigation of the effects of apigenin and curcumin showed that neither agent regulates hINV promoter activity. Moreover, concomitant application of either apigenin or curcumin with EGCG causes suppression of the EGCG-dependent increase in pINV-241 promoter activity. Additional studies indicate that both apigenin and curcumin inhibit EGCG-dependent activation of endogenous hINV protein expression. Taken together, these studies

suggest that apigenin and curcumin can oppose the differentiation-promoting action of EGCG in cultured normal human keratinocytes.

Regulation of AP 1 transcription factor function

AP1 is known to regulate involucrin gene expression in human epidermis and in keratinocytes. Our transfection studies using progressively truncated hINV promoter segments showed that the EGCG response element is located in the proximal regulatory region (PRR) spanning nucleotides −128/−110, and mutation analysis showed that the AP1-1 site, located within this region, is required for this regulation (Balasubramanian et al., 2002; Balasubramanian and Eckert, 2004; Balasubramanian et al., 2006). Based on these studies, we concluded that EGCG activates hINV gene expression via an AP1 transcription factor-dependent pathway, and that apigenin and curcumin oppose this action (Balasubramanian et al., 2002; Balasubramanian and Eckert, 2004; Balasubramanian et al., 2006). Immunoblot studies confirm that EGCG enhances the level of the AP1 factors including Fra-1, Fra-2, c-fos, fosB, Jun-B, Jun-D and c-jun. Moreover, gel supershift analysis reveals that EGCG increases binding of Fra-1 and Jun-D to the hINV promoter AP1-1 site (Balasubramanian et al., 2002; Balasubramanian and Eckert, 2004; Balasubramanian et al., 2006).

The above studies suggest that apigenin and curcumin may inhibit differentiating agent-dependent hINV gene expression by suppressing AP1 factor function. To understand these effects, we treated keratinocytes with TPA, a keratinocyte differentiation agent that increases hINV gene expression by increasing AP1 factor level and AP1 factor binding to the hINV promoter AP1 sites (Welter et al., 1995). Both apigenin and curcumin suppress the TPA-dependent increase in the level of c-jun, junB, junD, Fra-1, Fra-2 and fos B, and this is associated with decreased AP1 factor binding to the hINV promoter AP1-1 site and reduced promoter activity (Balasubramanian et al., 2002; Balasubramanian and Eckert, 2004; Balasubramanian et al., 2006). Proteasome function has been implicated in the regulation of transcription factors in human keratinocytes (Balasubramanian and Eckert, 2004). Our recent results reveal that treatment with MG132, a proteasome inhibitor, reverses the apigenin- and curcumin-dependent reduction in AP1 transcription factor levels, suggesting that proteasome function is required for apigenin and curcumin action (Balasubramanian et al., 2006; Balasubramanian and Eckert, 2006).

Differential regulation of novel protein kinase c

As noted above, involucrin expression is controlled by a novel protein kinase c (nPKC), Ras, MEKK1, MEK3 signaling cascade which ultimately acts to increase AP1 factor level and binding to the hINV promoter AP1-1 site to activate hINV gene expression (Efimova and Eckert, 2000; Efimova et al., 1998; Efimova et al., 2002; Efimova et al., 2003; Efimova et al., 2004; Eckert et al., 2004; Welter et al., 1995). Our studies indicate that EGCG activates this pathway to increase hINV gene expression, and that this response can be inhibited using BIS-IM, an agent that inhibits all PKC isoforms (Balasubramanian et al., 2002). Additional recent results indicate that novel PKC isoforms increase hINV promoter activity, that EGCG significantly increases nPKCs-dependent activation of hINV gene expression (unpublished), and that treatment with apigenin or curcumin inhibits the nPKC-dependent hINV promoter activation. This finding is consistent with other reports showing that apigenin and curcumin inhibit PKC action (Lin et al., 1997).

We recently reported the effects of apigenin and curcumin treatment on PKCδ phosphorylation. Altered PKCδ activity in human keratinocytes is associated with changes in phosphorylation at tyrosine-311 (Eckert et al., 2004; Efimova et al., 2004; Konishi et al., 2001). Increased PKCδ-Y311 phosphorylation is positively associated with TPA treatment, and is correlated

with increased expression of AP1 transcription factors and increased hINV promoter activity. Co-treatment with apigenin suppresses the TPA-dependent increase in promoter activity and phosphorylation analysis reveals that apigenin-treated cells display reduced levels of PKCδ-Y311 phosphorylation when compared to untreated cells (Balasubramanian et al., 2006). That the chemopreventive agent-dependent reduction in Y311 phosphorylation is biologically important is supported by data showing that a PKCδ tyrosine 311 mutant, PKCδ-Y311F, in which the tyrosine is converted to phenylalanine, has reduced ability to regulate AP1 factor levels and increase hINV promoter activity when compared to wild-type PKCδ (Balasubramanian et al., 2006).

Differential regulation of MAPK signaling

In human keratinocytes Ras and MEKK1 are activated in response to nPKC activation (Efimova et al., 1998). Expression of dominant-negative forms of Ras and MEKK1 suppress the EGCG-dependent increase in hINV promoter activity. This indicates that Ras and MEKK1 activity are required for the EGCG-dependent increase in hINV gene expression (Balasubramanian et al., 2002; Balasubramanian and Eckert, 2004). In contrast, apigenin and curcumin treatment inhibit the caRas- and MEKK1-dependent increase in promoter activity. Biochemical studies reveal that p38δ is activated (phosphorylated) following EGCG treatment (Balasubramanian et al., 2002), and that treatment with apigenin or curcumin suppresses this activation. Thus, EGCG and apigenin/curcumin appear to differentially regulate MAPK signaling in normal keratinocytes.

Differential regulation of cell morphology and apoptosis

We have also reported the impact of chemopreventive agent treatment on normal keratinocyte proliferation and apoptosis. Our findings indicate that EGCG, apigenin or curcumin treatment suppresses keratinocyte proliferation. Thus, in contrast to the opposing impact of EGCG versus curcumin/apigenin treatment on keratinocyte differentiation, all three compounds act to suppress proliferation. Interestingly, the reduction in cell number is associated with differential morphological changes. Normal human keratinocytes grow as loose non-structured colonies. In contrast, treatment with EGCG results in highly adherent flattened colonies, and apigenin treatment causes a circular flattened morphology. Moreover, the apigenin-associated morphology predominates in cells that are co-treated with TPA + apigenin or EGCG + apigenin (Balasubramanian et al., 2006). The apoptotic response is also varied. Treatment with EGCG or apigenin does not induce apoptosis in normal human keratinocytes (Balasubramanian et al., 2005; Balasubramanian et al., 2002; Balasubramanian and Eckert, 2004). However, curcumin causes a strong apoptotic response which is characterized by cell rounding, substrate detachment, increased number of cells with sub-G1/S DNA content, altered expression of cell cycle regulatory proteins, altered Bax/Bcl-xL expression and caspase activation in normal human keratinocytes (Balasubramanian and Eckert, 2006).

Summary

The present studies support several general conclusions regarding the impact of chemopreventive agents on normal human keratinocytes. *First*, these studies show that EGCG can promote keratinocyte differentiation. This is interesting, since promoting keratinocyte differentiation may be a mechanism whereby mutagenized cells can be removed from the epidermis. *Second*, chemopreventive agents can have opposing actions. For example, as summarized in Table 1, EGCG treatment stimulates keratinocyte differentiation but apigenin or curcumin treatment suppresses differentiation. Moreover, one chemopreventive agent may antagonize the action of another. For example, apigenin and curcumin inhibit the EGCG-dependent increase in keratinocyte differentiation (Balasubramanian and Eckert,

2004;Balasubramanian et al., 2006). This finding is perhaps not surprising, considering the structural differences among these compounds. *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 (Nomura et al., 2000;Dong et al., 1997;Barthelman et al., 1998;Chung et al., 1999). This suggests that the activity and mechanism of chemopreventive agent action may change during disease progression. *Fourth*, chemopreventive agents may simultaneously antagonize and synergize. Thus, while EGCG treatment increases AP1 and C/EBP factor levels in keratinocytes, this action is inhibited by curcumin and apigenin. However, in contrast, both agents act to suppress keratinocyte proliferation. This suggests that although EGCG and curcumin, for example, have opposing action on differentiation, they may still be effective when used together because of they suppress proliferation. An important lesson derived from these experiments is that not all chemopreventive agents are created equal and that it will be important to consider how simultaneous use of multiple chemopreventive agents may be used to enhance the therapeutic response.

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Abbreviations

EGCG
(−)-epigallocatechin-3-gallate

TPA
12-*O*-tetradeconylphorbol-13-acetate

hINV
human involucrin

KSFM
keratinocyte serum-free medium

PKC
protein kinase C

nPKC
novel PKC

MAPK
mitogen-activated protein kinase

AP1
activator protein 1

C/EBP
CAATT enhancer binding protein

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Table 1

Differential regulation of normal human keratinocytes by chemopreventive agents

Agent	EGCG	Apigenin	Curcumin
Effective Concentration μM	10 – 40	10 – 20	10 – 20
Proliferation	Suppresses	Suppresses	Suppresses
Differentiation	Activates	Suppresses	Suppresses
Apoptosis	None	None	Activates
Cell Morphology	Adherent colonies & web arrays	Circular flattened	Smaller, less densely packed and detached
Involucrin Expression	Activates	Inhibits differentiation agent-dependent increase	Inhibits differentiation agent-dependent increase
AP1 factor level and activity	Increases	Reduces	Reduces
C/EBP factor level	Increases	Reduces	Reduces
nPKC action	Increases	Suppresses	Suppresses
p38δ	Activates	Suppresses	Suppresses
Caspase cleavage and activity	No effect	No effect	Activates
Sub-G1 cells	Very few	Very few	Many