Gene expression changes induced by green tea polyphenol (−)-epigallocatechin-3-gallate in human bronchial epithelial 21BES cells analyzed by DNA microarray

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Abstract

Many studies suggest green tea is a cancer chemopreventive agent. This effect has been attributed to its major constituent (−)-epigallocatechin-3-gallate (EGCG). EGCG is also observed to have cytotoxic anticancer effects, especially when used in combination with certain chemotherapeutic agents. The biochemical actions of EGCG in chemoprevention and anticancer effects have been studied; however, the mechanisms of action are not clearly understood. We show here by expression genomics the effects of EGCG (25 μmol/L) in the Ha-ras gene transformed human bronchial epithelial 21BES cells. We found induction of temporal changes in gene expression and the coalescence of specific genetic pathways by EGCG. In this experimental system, hydrogen peroxide (H2O2) was produced. By treating cells with EGCG in the presence or absence of catalase, we further distinguished gene expression changes that are mediated by H2O2 from those that are H2O2 independent. Many genes and cellular pathways, including genes of the transforming growth factor-β signaling pathway, were H2O2 dependent because the effects were abolished by catalase. Gene expression changes that were not affected by catalase included those of the bone morphogenetic protein signaling pathway, peptidylprolyl isomerase (cyclophilin)–like 2, alkylated DNA repair enzyme alkB, polyhomeotic-like 2, and homeobox D1. We show further that EGCG and H2O2 differentially transactivated the bone morphogenetic protein and the transforming growth factor-β response element promoter reporters, respectively, thus confirming results from DNA microarray analysis. The elucidation of gene expression changes between H2O2-dependent and H2O2-independent responses helps us better understand the cancer chemopreventive and anticancer actions of EGCG.

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Introduction

Some epidemiologic studies suggested that green tea, a widely consumed beverage, lowers the risk of certain types of cancers (1–3). Whereas tea contains several bioactive chemicals, it is particularly rich in catechins, of which (−)-epigallocatechin-3-gallate (EGCG) is most abundant and most active (1). Catechins and their metabolites are thought to contribute to the beneficial health effects ascribed to tea. In addition to its cancer preventive effects, EGCG has also been shown to have anticancer effects in various experimental systems including those of cervical, colon, stomach, and prostate cancers (1,4–6). Recently, synergistic cytotoxic anticancer effects was also obtained when EGCG is applied in combination treatment with certain anticancer drugs (7–10). These chemosensitivity tests suggest that EGCG has a potential in adjuvant cancer therapy.

Many mechanisms have been proposed for the chemopreventive and anticancer activities of EGCG. These include the inhibition of telomerase, mitogen-activated protein kinase (MAPK), activator protein-1, nuclear factor-κB, binding of epidermal growth factor to its receptor, angiogenesis, and activation of apoptosis (11–18). These proposals suggest that EGCG may have pleiotropic effects in cells. Nevertheless, the targets for the chemopreventive and anticancer effects of EGCG have not been clearly elucidated.

EGCG have multiple biological effects by acting on different targets directly or indirectly. Further confounding the results is the generation of hydrogen peroxide (H2O2) when EGCG is added to cell culture system (19–23), thus raising questions about whether the reported biochemical effects of EGCG are attributable to EGCG directly or to H2O2. In our previous studies with human lung cancer cell lines and the Ha-ras gene transformed human bronchial epithelial 21BES cell line, EGCG was found to inhibit cell growth and to induce apoptosis (19,20). The induction of apoptosis by EGCG in these cell lines was either partially or completely abolished in the presence of catalase, which decomposes H2O2.

Recent advances in gene expression profiling by DNA microarray have enabled genome-wide elucidation of the functional genomics of genes involved in various cellular
EGCG-Induced Gene Expression Changes

pathways (24–30). We have shown recently that surveying genome-wide gene expression changes facilitates the identification of specific pathways essential for the tumor promoter 12-O-tetradecanoylphorbol-13-acetate–mediated differentiation in the human leukemia HL-60 cells (26). DNA microarray expression profiling of genes affected by EGCG has also been conducted with limited number of expressed sequence tag probes (5, 31, 32) and a few sampling of time points (either one or three time intervals) following EGCG treatment.

To further understand the anticancer and chemopreventive mechanisms of EGCG, we determined effect of EGCG on genome-wide expression profiles in 21BES cells. Our results show distinct temporal changes in gene expression induced by EGCG. Addition of catalase, which catalyzes the decomposition of \( \text{H}_2\text{O}_2 \), abolished a notable number of these gene changes such as transforming growth factor (TGF)-\( \beta \)2, SMAD3, and TSC22. Gene expression changes that are not inhibited by catalase include bone morphogenetic protein (BMP) type II receptor (BMPR2), peptidyl-prolyl isomerase (cyclophilin)–like 2, alkylated DNA repair enzyme \( \text{alkB} \), and STAT5B. We show further that \( \text{H}_2\text{O}_2 \) transactivated TGF-\( \beta \) response element promoter activity and this transactivation was blocked by catalase, whereas EGCG transactivated BMP response element promoter and this effect was not influenced by catalase.

Materials and Methods

Cell Culture, Chemicals, and \( \text{H}_2\text{O}_2 \) Assay

The Ha-ras gene transformed human bronchial epithelial 21BES cell line was obtained from American Type Culture Collection (Rockville, MD) and maintained in LHC-9 medium (Biofluids, Rockville, MD). The purified green tea polyphenol EGCG was provided by Thomas J. Lipton Co. (Englewood Cliffs, NJ) and catalase was from Sigma Chemical Co. (St. Louis, MO).

Measurement of \( \text{H}_2\text{O}_2 \) production was conducted as described previously (21). In brief, EGCG (25 \( \mu \text{mol/L} \)) was added to LHC-9 medium either in the presence or in the absence of cells and with or without catalase (30 units/mL). At different time points, the amounts of \( \text{H}_2\text{O}_2 \) in medium were analyzed using an Amplex Red \( \text{H}_2\text{O}_2 \) assay kit (Molecular Probes, Eugene, OR).

DNA Microarray

DNA arrays were custom printed on 3 \( \times \) 10 in. nylon membrane and contained \( \sim \)10,692 DNA elements composed of expressed sequence tags that either corresponded to human transcripts with known function in the Genbank database (7,117) or anonymous (3,575). RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. Integrity of isolated RNA was examined by gel electrophoresis.

Target Synthesis, Array Hybridization, Image Processing, and Statistical Analysis

cDNA targets were synthesized from the isolated total RNA with \( ^{33}\text{P}-\text{dCTP} \) by oligodeoxythymidylic acid–primed polymerization using Superscript II reverse transcriptase (Life Technologies, Rockville, MD) as described previously (26). \( ^{33}\text{P}-\text{dCTP} \)-labeled targets were hybridized to the membrane arrays, washed, and exposed on phosphorimage screen for \( \sim \)15 hours and scanned on a Typhoon PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Scanned images of microarrays were analyzed using ImaGene (Biodiscovery, Danville, CA) and the output intensity data were further analyzed using custom statistical software.

Array data analysis was done as described previously (26). In brief, normalized expression data for each gene were analyzed by regression models to fit polynomial functions of the logarithm of time up to the third degree. A subset of genes with \( >2 \)-fold changes for at least two consecutive time points and with significant coefficients of the highest degree (i.e., linear, quadratic, or cubic) at the significance level of 2% (to control possible type I error rate from multiple comparisons) was included in the polynomial regression analysis. Local regression models were also used to fit quadratic polynomials of the logarithm of time as before (26). For the selected genes, the patterns of gene expression were log transformed, centered by median, and subjected to cluster analyses by centered correlation and average linkage as the similarity/distance metric using the hierarchical cluster algorithm in Cluster and TreeView software suite (33). The entire microarray data set is available for searches at http://www.ncbi.nlm.nih.gov/geo under the data series accession no. GSE#.

Transactivation of TGF-\( \beta \) and BMP Response Element Promoter Reporter

21BES cells were transfected with either TGF-\( \beta \) (3TP-Lux; ref. 34) or BMP response element (MSX; ref. 35) promoter luciferase reporter plasmids and treated with either EGCG or \( \text{H}_2\text{O}_2 \) in the presence or absence of catalase 24 hours post-transfection. Luciferase assays were conducted in three independent transfections and normalized with Renilla luciferase activity.

Real-time Quantitative Reverse Transcription-PCR Validation of Gene Expression

Real-time quantitative reverse transcription-PCR was done to analyze the expression of BMPR2, CDH3, DUSP5, FKBP5, FOXP1, and ZNF184. Total RNA (\( \sim \)35 ng) from untreated control and EGCG-treated cells as described above in the microarray studies were employed for the amplification. RNA from Raji cells (Applied Biosystems, Foster City, CA) was used as internal control for the expression of ZNF184 and the human placental RNA was used for all other genes. cDNA synthesis and real-time PCR amplification were conducted using Taqman assay. The minor groove binder probe was labeled by 5′-end FAM as reporter. Amplifications were conducted in triplicate using ABI Prism 7000 sequencing detector.

Results

Consistent with our previous report (20), we show here that exposing the Ha-ras gene transformed human bronchial...
epithelial 21BES cell line to EGCG (25 mol/L) inhibited cell growth and caused cell death as determined morphologically by light microscopy (Fig. 1). Also consistent with previous observations (20, 21), we found a rapid but transient production of H2O2 when EGCG was added to the cell culture, with a peak concentration of ~3 mol/L (Fig. 2). Addition of catalase (30 units/mL) to the cell culture system markedly reduced the production of H2O2. Controls without EGCG had much lower levels of H2O2 (0.2–0.4 mol/L) in the culture medium and catalase further decreased the H2O2 level (Fig. 2). The results suggest that H2O2 is generated by the auto-oxidation of EGCG in the culture medium and the addition of catalase effectively reduced H2O2 level. The presence of catalase delayed the EGCG-induced onset of cell death (Fig. 1, K, L, O, and P), suggesting that H2O2 is in part involved in the EGCG-induced apoptosis. This is consistent with our previous results as well as with others in that addition of exogenous catalase failed to completely prevent the EGCG-induced cytotoxicity and cell growth in the tumor cells (20, 36).

We determined the time-dependent gene expression profiles of cells after exposure to EGCG. RNAs were isolated from 21BES cells treated with EGCG (25 μmol/L) in the presence or absence of catalase (30 units/mL) at 0, 0.25, 0.5, 1, 2, 3, 6, 12, 15, 24, 36, and 48 hours and compared with untreated controls. Samples were labeled by reverse transcription and hybridized to custom-printed cDNA arrays with 10,692 expressed sequence tags printed on nylon membranes (see Materials and Methods). Normalized data were analyzed by regression models and the expression levels for each gene were fitted to polynomial functions relative to the logarithm of time up to

Figure 1. EGCG induced apoptosis in 21BES cells. Onset of cell death following EGCG (25 μmol/L) treatment either in the presence (M–P) or in the absence (I–L) of catalase (30 units/mL) was observed by light micrography compared with controls without (A–D) or with (E–H) catalase at the following time points: 1 hour (A, E, I, and M), 12 hours (B, F, J, and N), 36 hours (C, G, K, and O), and 48 hours (D, H, L, and P).
Figure 2. Formation of H2O2 in conditioned tissue culture medium from auto-oxidation of EGCG. Following EGCG addition to cell culture, conditioned medium was collected and assayed for H2O2 as described in Materials and Methods at 0, 0.25, 0.5, 1, 2, 3, 6, 12, 15, 24, 36, and 48 hours. ○, untreated control; ●, catalase (30 units/mL); △, EGCG (25 μmol/L); ●, catalase (30 units/mL) + EGCG (25 μmol/L).

The temporal gene changes following EGCG treatment occurred as early as 15 minutes with the early-response genes, with increased expression returning to basal levels by 3 to 6 hours (Fig. 3C). A second cascade of gene induction followed between 3 and 10 hours and the late-response cluster between 12 and 36 hours post–EGCG treatment. In the early-response cluster, the down-regulated genes included sex determining region Y-box 12 (SOX12), peptidylprolyl isomerase (cyclophilin)–like 2 (PPIL2), zinc finger protein 33A (ZNF33A), inositol hexophosphate kinase 1 (IHPK1), BMPR2, and alkylation repair alkB homologue (ALKBH) and the up-regulated genes included ubiquitin-activating enzyme E1C (UBEIC), FMS-related tyrosine kinase 1 (FLT1), and basic fibroblast growth factor 2 (FGF2; Fig. 3B). In the intermediate-response gene cluster, we found characteristic induction of transcription regulators [TGF-β-stimulated protein (TSC22), polyhomeotic-like 2 (PHC2), and homeobox D1 (HOXD1)] and genes that may have roles in apoptosis [thromboxane A2 receptor (TXA2RI), tumor necrosis factor receptor gene superfamily member 6 (TNFRSF6), and MAPK activating death domain protein (MADD)] as well as cell growth inhibition and differentiation [eukaryotic translation initiation factor 4E (EIF4E), HOXD1, and TSC22; Fig. 3B]. Genes in the late-response cluster seemed to have roles in various cellular pathways including matrix metallopeinase-9 (MMP9), caveolin 1 (CAV1), and cytochrome P450 CYP3A7 but notably an absence of transcription factors (Fig. 3B).

Because the addition of EGCG caused production of H2O2, which is a known inducer of gene expression (37–39), it is necessary to distinguish between the genes that are H2O2 dependent and the genes that are H2O2 independent. Therefore, we treated 21BES cells in the presence of catalase (30 units/mL) to abolish or minimize the effects of H2O2. Our results showed that some of the changes in gene expression were abolished by the addition of catalase (Fig. 4A), whereas other changes were unaffected (Fig. 4B), thus suggesting that the former group of genes is mediated by H2O2.

Notably, a significant number of the H2O2-affected genes (that were abolished by catalase) were early-response genes or biphasic genes that expressed in a cyclical fashion (Fig. 4A). In contrast, most genes, with expression not blocked by catalase, were of either the intermediate-response or the late-response clusters (Fig. 4B). These results suggest that the acute response in gene expression on addition of EGCG may be mostly mediated by H2O2.

Coordinated changes in the expression of genes with common pathways or functions were also observed (Fig. 4A). For example, there was the activation of components of the TGF-β signaling pathway (40) including TGF-β1 and TGF-β2, the TGF-β-stimulated protein TSC22, SMAD2, SMAD3, and SMAD6, and SMAD anchor for receptor activation (SARA) and some downstream targets including fibronectin, fibronectin receptor, laminin, laminin receptor, etc. (Fig. 4A). Interestingly, treating cells with catalase can abolish the activation of these genes (Fig. 4A), suggesting that these changes are mediated by H2O2. In contrast, decreased expression of a gene cluster of the BMP signaling pathway (41) including its BMPR2, SMAD7, some components of the MAPK pathway, Fk506 binding protein 5, cullin 5, etc., were not blocked by catalase (Fig. 4B). These results suggest that the BMP signaling pathway and components of its downstream target genes are affected by EGCG independent of H2O2.
Figure 3. TreeView displays and polynomial regression analysis of temporal changes in gene expression by EGCG. DNA microarray expression data of EGCG (25 μmol/L)-treated 21BES cells in the presence or absence of catalase (30 units/mL) compared with untreated control with or without catalase were normalized and filtered as described in Materials and Methods. A, selected subset of genes was log transformed, centered by median, and subjected to cluster analyses by centered correlation and complete linkage using the hierarchical cluster algorithm and display using the TreeView software suite. B and C, temporal patterns of gene expression were obtained by polynomial regression analysis and the genes clusters were displayed with TreeView and histograms.
was observed with H$_2$O$_2$ either in the absence or in the presence of catalase. We found transactivation of the TGF-$eta$-responsive promoter reporter (3TP-Lux) by H$_2$O$_2$, which was modestly inhibited by catalase (Fig. 5B). No significant transactivation of TGF-$eta$ response element promoter reporter, however, was observed with EGCG (Fig. 5B). These results suggest that EGCG and H$_2$O$_2$ can specifically modulate transcription of genes of the BMP and TGF-$eta$ signaling pathways, respectively.

Using RNA samples derived from the microarray expression studies above, we further validated the expression of BMPR2, CDH3, DUSP5, FKBP5, FOXP1, and ZNF184 by real-time quantitative PCR. These genes were chosen from the cluster of the BMP signaling pathway. Our results suggested that the expression pattern of these genes agrees with those observed in the microarray experiments between untreated and EGCG-treated cells (Fig. 6).

Discussion
Our results showed that, on addition of EGCG to 21BES cells, altered gene expression, cell growth inhibition, and apoptosis were observed (Fig. 1). More importantly, we were able to dissect the gene expression profiles into either H$_2$O$_2$-dependent or H$_2$O$_2$-independent expression patterns by treating 21BES cells with EGCG in the presence of catalase (Fig. 4). Most of the H$_2$O$_2$-mediated gene expression (Fig. 4A) occurred either as early-response or as intermediate-response genes, whereas a significant number of H$_2$O$_2$-independent genes occurred later in either the intermediate-response or the late-response clusters (Fig. 4B).

The presence of catalase abolished the H$_2$O$_2$ induction of apoptosis genes (TXA2R, TNFRSF6, and MADD; Fig. 2B) and slowed down the rate of apoptosis. Cell death still occurred in 21BES cells with a delay of ~12 to 24 hours, suggesting that EGCG also induced H$_2$O$_2$-independent apoptosis events.

Detailed kinetics analysis of gene expression changes showed that EGCG causes distinct temporal activation of gene expression that can be distinctly separated into early-response, intermediate-response, and late-response clusters of gene expression (Fig. 3). These temporal gene changes are coordinately modulated with transcription factors (TSC22, HOXD1, and PHC2), cell differentiation (EIF4E, PHC2, and HOXD1), and cell death regulators (TXA2R, TNFRSF6, and MADD) altered in the early-response and intermediate-response clusters followed by the activation of genes with a variety of cellular functions.
functions that are downstream targets of the early-response and intermediate-response genes. These patterns of gene changes suggest a hierarchical and coordinated regulation of gene expression by EGCG that are consistent with cell growth inhibition and the activation of apoptosis.

Among many of the gene expression changes observed, we found that those involving TGF-β and BMP signaling pathways were most interesting and selected for additional study. The EGCG-induced down-regulation of the BMP signaling pathway was not influenced by the presence of catalase (Fig. 3). This effect was confirmed by reporter assay, demonstrating that EGCG repressed BMP response element promoter activity, and the suppression was not affected by catalase (Fig. 5A). However, most of the genes of the TGF-β pathway can be abolished by catalase, suggesting that their induction is mediated by H₂O₂. We also confirmed by promoter reporter transactivation assay that H₂O₂ stimulated TGF-β response element promoter and the activation was inhibited by catalase (Fig. 5B). Unexpectedly, EGCG did not stimulate the TGF-β response element promoter reporter. We speculate that, because gene expression changes activated by EGCG are temporal sensitive, harvesting the cells for luciferase assays at 24 hours post-transfection and treatment with EGCG may not coincide with its temporal onset of induction of gene expression. Nevertheless, our results are consistent with the hypothesis that, in 21BES cells, the induction of the TGF-β signaling pathway is mediated by H₂O₂, whereas the suppression of the BMP signaling pathway is H₂O₂ independent.

BMPs belong to the TGF-β family and have been identified as factors that stimulate bone formation in vivo, but BMPs are multifunctional molecules regulating growth, differentiation, and apoptosis in various target cells (41). The observation that the mRNA levels of the BMPR2, SMAD7, and components of the MAPK pathway (MAP3K8 and MAPK-APK3; ref. 42; Fig. 4B) are decreased by EGCG suggests that the BMP signaling pathway is down-regulated by EGCG. It has been shown that BMPR2 stimulates the MAPK pathway by a BMPR2-induced up-regulation of mRNA for ERK1 and ERK2 (43). Thus, suppression of the BMP pathway by EGCG may result in a decrease of the MAPK pathway. In addition, EGCG can also inhibit the enzymatic activity of MAPK (14). Together, these effects may contribute to the growth inhibitory effects of EGCG. Activation of the BMP pathway, which also activates apoptosis as shown in previous studies (41), is consistent with the EGCG-induced cell apoptosis observed in our study (Fig. 1A).

H₂O₂ has been shown previously to induce gene expression (37–39). Its downstream signaling events include
calcium mobilization and protein phosphorylation. A previous microarray expression profiling study showed that H2O2 induced a large number of gene changes (37) and some of these gene changes were also observed in our study. These include glutathione peroxidase 2, early growth response 1, CHK1 homologue, SMURF2 (an E3 ubiquitin ligase), Jun, cyclin A2, NADP+-dependent malic enzyme 1, etc., representing genes from diverse biochemical and functional pathways (Fig. 4A). However, there are also many differences in gene expression profile between ours and this previous study mainly due to the rather high concentration of exogenous H2O2 (500 μmol/L) used to treat the cells (37). The distinct dynamic patterns of early-response, intermediate-response, and late-response changes in gene expression in our study were not pursued by Chuang et al. (37). Clearly, detailed time course and dose-dependent analyses with multiple, closely spanned time points or dosages should be conducted in the future to gain further understanding of the gene expression pattern of H2O2.

The H2O2 formed on addition of EGCG to a cell culture system is probably due to the auto-oxidation of EGCG because of the presence of rather high oxygen partial pressure (160 mm Hg). The oxygen partial pressure is 100 to 150 mm Hg in lung alveoli, 85 to 90 mm Hg in arterial blood, and ~40 mm Hg in mixed venous blood (44). Therefore, whether cellular EGCG causes the production of H2O2 in vivo is unknown and remains to be studied.

In the present study, the EGCG (25 μmol/L) used was following that applied in our previous studies (20). This concentration is higher than those detected in the plasma samples of humans and rodents following tea ingestion (1). Therefore, it remains to be determined whether the presently observed effects can be recapitulated in vivo after dietary or pharmacologic doses of green tea or EGCG. Nevertheless, a high concentration of EGCG can be delivered to the oral cavity; thereafter, high salivary concentrations of EGCG can be observed (45). In the oral cavity, the salivary oxygen partial pressure may approximate that in the cell culture.

In summary, gene expression profiling analysis in our study revealed temporal changes in gene expression profiles on addition of EGCG to 21BES cells. The distinction between H2O2-dependent and H2O2-independent pathways are important because the former, such as the effect on the TGF-β signaling pathway, may or may not occur in vivo. Gene changes not affected by catalase such as those of the BMP signaling pathway may be more important for EGCG-induced cell growth inhibition and death. Further studies will be required to determine whether BMP and its receptors as well as other gene expression changes significantly contribute to the cancer chemopreventive and anticancer effects of EGCG.

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