Published in final edited form as:

Mol Nutr Food Res. 2008 October ; 52(10): 1182–1192. doi:10.1002/mnfr.200700499.

The Modulation of Endothelial Cell Gene Expression By Green Tea Polyphenol-EGCG

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Abstract

Human and animal studies have shown that green tea consumption is associated with a reduced risk of some cancers. This has been attributed to its polyphenol components, in particular (–)-epigallocatechin gallate (EGCG). In addition to be a cancer chemopreventive agent, EGCG inhibits angiogenesis, thus reducing tumor growth and metastasis. We tested EGCG modulation on the gene expression profile of endothelial cells stimulated by VEGF using Affymetrix microarrays. A total of 421 genes were up-regulated and 72 genes were down-regulated at the false discovery rate of 5% by VEGF, EGCG, and EGCG pretreatment followed by VEGF stimulation. The changes in the expression of several pivotal genes were validated by real-time PCR. Furthermore, we have identified two signaling pathways (Wnt and Id) involved in cell proliferation were modulated by EGCG treatment, suggesting the negative regulation of EGCG on cell proliferation. Our results also indicate that the anti-angiogenesis effect of EGCG is partially mediated through its broad inhibition on endothelial cell proliferation. Our data further support earlier observations that the anticancer effect of EGCG is mediated through changes in the expression of genes that are associated with cell proliferation.

Keywords

EGCG; endothelial cell; microarray; MAPP analysis; proliferation

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See also supplemental Tables 1–3 available as Online Supporting Material with the online posting of this paper at <http://jn.nutrition.org>

URL for signaling pathways:

Cholesterol biosynthesis pathway:

http://www.genmapp.org/HTML_MAPPs/Human/Hs_Contributed_20051123/metabolic_process-GenMAPP/Hs_Cholesterol_Biosynthesis/Hs_Cholesterol_Biosynthesis.htm.

Wnt pathway:

http://www.genmapp.org/HTML_MAPPs/Human/Hs_Contributed_20051123/cellular_process-GenMAPP/Hs_Wnt_signaling/Hs_Wnt_signaling.htm.

Id pathway:

http://www.genmapp.org/HTML_MAPPs/Human/Hs_Contributed_20060824/cellular_process-GenMAPP/Hs_Id_NetPath_5/Hs_Id_NetPath_5

INTRODUCTION

Epidemiological studies have shown that the consumption of green tea is associated with reduced risk of some cancers including gastric, esophagus and pancreas.^(1–4) Studies in animal models and *in vitro* cell culture system also suggest that green tea and green tea extract inhibit the development and progression of skin, lung, mammary gland, and gastrointestinal tumors.^(5, 6) Although the protective mechanisms of green tea have not been fully elucidated, it has been proposed that green tea extract inhibits cell proliferation and causes cell apoptosis *in vitro* by modulating signal transduction.⁽⁷⁾ The protective effect of green tea has been attributed to the biological activities of its polyphenol catechins content, in particular (–)-epigallocatechin gallate (EGCG), the major constituent in green tea extract, which has been shown to have significant anti-proliferative and anti-carcinogenic properties.⁽⁸⁾

In addition to anti-proliferative effects, green tea has been shown to inhibit tumor invasion and angiogenesis,⁽⁹⁾ which are the crucial steps for the growth and metastasis of all solid tumors. Angiogenesis, the formation of new blood vessels from the existing vessels, is involved in physiological processes such as wound healing as well as in pathological processes such as tumor growth and atherosclerosis.^(10, 11) We and others have shown that EGCG inhibits tubular structure formation of endothelial cells in culture via modulation of vascular endothelial growth factor (VEGF) signaling, including phosphorylation of VEGF receptor and vascular endothelial (VE)-cadherin, disruption of VEGF-induced VE-cadherin/ β -catenin complex, and inhibition of Akt phosphorylation and IL-8 production.^(12, 13) It has been reported that the anticancer activity of EGCG is also associated with the inhibition of invasion by suppressing the activity of urokinase^(14, 15) or the matrix metalloproteinases (MMPs).⁽¹⁶⁾ Thus, it appears that green tea EGCG may contribute to cancer prevention by reducing cell proliferation, tumor cell migration, and invasion, and by inhibiting angiogenesis. In order to gain further insights to the understanding of metabolic pathways that are affected by EGCG, we tested the effect of EGCG on genes expression in endothelial cells using Affymetrix microarrays system.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVEC) cells were purchased from Cambrex (Walkersville, MD). Cells from passage 6 were grown in 100 mm petri dishes. The cells were maintained with 2% fetal bovine serum (FBS) using certified EBM-2 medium supplemented with growth factor kit (EGM-TM2 SingleQuots, Cambrex), which provides optimal condition for HUVEC proliferation. At 80% confluency, cells were supplemented with or without EGCG (LKT Laboratories Inc., MN), 20 μ M in dimethyl sulfoxide (DMSO) for 24h. DMSO final concentration in medium was 0.02%. Since Abe and Sato⁽¹⁷⁾ showed a very active gene expression profile following 30 min stimulation of HUVEC with VEGF, we therefore chose to stimulate HUVEC with 50 ng/mL VEGF for 30 min. Then the cells were washed and harvested for RNA extraction.

Measurement of cell proliferation

HUVEC were grown in 6-well plates up to 60% confluency. EGCG at concentrations of 5, 10, 20 μ M and VEGF (final concentration 50ng/mL) were added into the cells culture medium and cells were incubated for 24h. The cells were trypsinized and cell suspension was prepared in 0.5 mL. 9 μ L was applied to hemocytometer and cell numbers per well were determined.

In vitro angiogenesis assay

24-well plates were coated with ice-cold growth factor-reduced Matrigel (250 μ L/well) (BD, MA). It was allowed to polymerize at 37°C for 30 min. Thereafter, 1 mL of a suspension of HUVEC (2×10^5 cells/mL), which had been treated with DMSO or 20 μ M EGCG in DMSO for up to 24 h, was seeded onto the Matrigel as described previously.⁽¹⁸⁾ The cells were maintained in EBM-2 medium for 48h in the presence or absence of VEGF (50ng/mL). Tube formation was assessed after 48h and quantified by determining the mean length and number of branching points in three randomly selected fields.

RNA extraction and preparation of biotin labeled cRNA for GeneChip analysis

RNA was extracted from cells subjected to the different treatments using the RNeasy Mini Kit (QIAGEN, Valencia CA) according to the manufacturer's protocol. Each experimental treatment was carried out in triplicate. cDNA was synthesized from 5 μ g total RNA by SuperScript reverse transcriptase (Invitrogen, Carlsbad CA). Then biotin-labeled cRNA was synthesized from cDNA with *in vitro* transcription. The cRNA samples were fragmented and hybridized to human U133A Array GeneChip (Affymetrix, Santa Clara CA) in the 45°C oven overnight, at 60 RPM. Microarrays were washed and stained according to the protocol provided by the manufacturer. The arrays were scanned using Agilent confocal laser scanner (Affymetrix) and analyzed using Affymetrix 5.0 software (Affymetrix).

Data analysis of Affymetrix microarrays

Affymetrix Human U133A Array contains 22,283 human probe sets. Each gene on the array is represented by a probe set consisting of 11 perfect match (PM) and 11 mismatch (MM) probe pairs. The quantitative estimate of expression of each probe set is the Signal (*Sig*) metric. *Sig* is computed using the One-Step Tukey's Biweight Estimate, which gives the weighted mean of the log (PM-MM) intensities for each probe set (Affymetrix Microarray Suite, Version 5.0). All microarrays were scaled to the same target signal using the "All Probe Sets" scaling option. A detection call (present, marginal or absent) is also given for each probe set. Only those genes that, 1) had signal over 25 (the mean intensity of all probe sets that had "absent" call), and 2) were called "Present" or "Marginal" in at least two of three replicates were included for statistical analysis. We used a Bayesian framework *t*-test (CyberT)⁽¹⁹⁾ to identify genes that show differential expression among the control and treatments. The CyberT statistic models the standard deviation as the function of signal intensity for each probe set using Bayesian framework. This approach was regarded as the most robust method to analyze Affymetrix microarray gene expression data⁽²⁰⁾. Furthermore, we corrected p-values from the Bayesian framework *t*-test for multiple tests using a false-discovery rate of 5% (*Q*-values ≤ 0.05) criterion for significance⁽²¹⁾.

Gene Ontology and Pathway Analysis

We used the GenMapp 2.0 and MappFinder 2.0 software package (www.GenMapp.org)^(22, 23) for gene ontology (GO) and pathway analysis. These resources contain most updated GO terms from GenMapp archives (www.GenMapp.org) and available pathways from KEGG (www.genome.jp/kegg),⁽²⁴⁾ as well as contribution of GenMapp users for humans. All the 12,271 genes that were detectable in gene expression and their *Q*-values were imported into the programs, and linked to the GO hierarchy and pathway profiles. Gene ontology in GenMapp program allows all measured genes to be organized into hierarchies in three aspects of their biological functions: biological processes, cellular components, and molecular functions. Each hierarchy is structured in multiple levels of parent-child relationships between its terms. For each GO node, MappFinder calculated the percentage of the genes that met the user-defined criterion (*Q* ≤ 0.05) among all the measured genes. We determined which GO terms and pathways (MAPP) were significantly

over-represented by the set of significant genes by computing *Z*-scores. *Z*-scores were computed by subtracting the expected number of genes in a GO term (or MAPP), meeting the criterion from the observed number of genes, divided by the standard deviation of the observed number of genes. A GO term or pathway with a *Z*-score greater than 1.96 (*P* 0.05) was considered to be statistically significant and of potential biological importance. GO terms describing fewer than two genes that met the user-defined criteria were not considered here as they were either too specific or too general⁽²³⁾. The filtered list of high *Z*-score GO terms was further annotated manually, to avoid redundant GO terms. When both a parent and a child GO term were presented in the list, the parent term was removed if its presence was due entirely to the genes meeting the criteria for the child term.

Validation of GeneChip data by real time PCR

The total RNA was used to validate selected number of responsive genes. The first strand cDNA synthesis was performed with the SuperScript II reverse transcriptase (Invitrogen) by using 1 µg total RNA. The target mRNA levels were determined by TaqMan real-time RT-PCR using the ABI prism 7700 Sequence Detection System (Applied Biosystems, Foster city CA) and the Ct method: the fold of suppression = $2^{Ct(\text{control}) - Ct(\text{treatment})}$. The TaqMan probe consisted of an oligonucleotide with a 5' reporter dye (FAM) and a downstream 3' quencher dye (NFQ). The reactions were performed on plates using adhesive seals as covers. TaqMan RT-PCR master mix Reagent kit (Applied Biosystems) in a total volume of 20 µL consisted of 3 mM MgCl₂, 0.2 mM/each dNTP, 200 nM probe, primers, and 1.25 U Amplitaq Gold. The PCR was programmed as follows: initial denature at 95°C for 10 min followed by 95°C for 15 sec, 60°C for 1 min, cycled 40 times. Each target was amplified in triplicates. The standard curve was constructed with serial dilutions of untreated endothelial cell RNA. A housekeeping gene β-actin was used as a reference for all of the samples. The primers were purchased from Applied Biosystems. They are premade primers and the catalog numbers are as follows: Hs00156507_m1(CENPE), Hs00357821_g1(ID1), Hs00183740_m1(DKK1), Hs99999903_m1(beta-actin). Tryptophanyl-tRNA synthetase (TrpRS): forward primer: GCATGTAGGTCACCTCATTCCATTT, reverse primer: GACCAAGGGCACGTAAATACATC, probe: CTGGAGCCACTTTGTG.

RESULTS AND DISCUSSION

1. Endothelial cell growth response to EGCG treatment

Cell proliferation—Cell proliferation modulated by EGCG pre-incubation was measured with and without VEGF stimulation. EGCG pre-incubation with and without VEGF stimulation dose-dependently inhibited HUVEC proliferation (Figure 1).

In Vitro Angiogenesis—Endothelial cell growing on Matrigel coated plates showed tube formation, which represented the angiogenesis capacity of endothelial cells *in vivo*. The cells without VEGF stimulation, due to the presence of some growth factor in Matrigel, showed formation of tubes. VEGF stimulation increased tube formation by 15%. Both stimulated and unstimulated tube formations were inhibited by pre-incubation of endothelial cells with EGCG (20 µM) for 24 h (Figure 2).

2. Gene expression responses to EGCG treatment in endothelial cells

Human U133A microarray contains 14,500 well-characterized human genes, of which the expressions of 8,400 genes were detected from endothelial cells in our experiment. A total of 421 genes were up-regulated and 72 genes were down-regulated (*Q* 0.05) by VEGF, EGCG, and EGCG pretreatment followed by VEGF stimulation (Table 1). We were specifically interested in the genes and functions that were affected by EGCG treatments, in particular, when cells were stimulated with VEGF.

The effects of VEGF on endothelial cells—In accordance with previous works^(17, 25, 26), our study has shown some typical regulatory effects of VEGF. Gene Ontology (GO term) analysis identified the impact of VEGF on several biological processes including activation of NF- κ B-inducing kinase, anti-apoptosis, angiogenesis and induction of positive chemotaxis (Table 2). Molecular functions such as chemokine and cytokine activities were also regulated by VEGF enrichment *in vitro* (Table 2). Similarly, MAPP pathway analysis has identified several pathways that have been regulated by VEGF stimulation, including apoptosis, adipogenesis, cytokine and inflammatory responses, and proliferation (Table 3).

The effects of EGCG on endothelial cells—Under EGCG treatments, there were 14 genes up-regulated and 14 genes down-regulated compared to the control group as calculated by both Bayesian *t*-test and Q-value correction (Table 1 and Supplemental Table 1). (Supporting supplemental Tables 1–3 are available as Online Supporting Material with the online posting of this paper at <http://jn.nutrition.org>).

To further elaborate the EGCG effect on modulation of cell proliferation, VEGF was added to stimulate cell growth (EGCG+VEGF treatment). Under this treatment, 116 genes were significantly up-regulated and 7 genes were down regulated compared to the group with EGCG alone (Table 1 and Supplemental Table 2). However, there was no differential gene expression when we compared the cells treated with EGCG+VEGF to the cells only treated with VEGF (Table 1).

Furthermore, based on GO analysis we found that EGCG exhibit strong regulatory effects on three biological processes: *cholesterol biosynthesis*, *microtubule activities* and *inhibition of cell proliferation* (Table 4 and Supplemental Table 1). As identified by MAPP finder (Table 3), 3 out of 15 genes (20%) in cholesterol biosynthesis pathway, have shown differential expressions: Isopentenyl-diphosphate delta isomerase, dehydrocholesterol reductase and sterol-C4-methyl oxidase-like genes (please see the pathway cholesterol biosynthesis at this site: (http://www.genmapp.org/HTML_MAPPs/Human/Hs_Contributed_20051123/metabolic_process-GenMAPP/Hs_Cholesterol_Biosynthesis/Hs_Cholesterol_Biosynthesis.htm)). The expressions of all these 3 genes were increased by EGCG treatment (Table 4, Supplemental Table 1). The increase of dehydrocholesterol reductase and sterol-C4-methyl oxidase-like genes would likely contribute to the synthesis of cholesterol. The significance of this effect of EGCG in endothelial cells warrants further investigation.

Negative regulation of EGCG on endothelial cell proliferation and angiogenesis—The negative regulation of EGCG on endothelial cell proliferation in this study (Table 4) appears to have a profound biological significance. TGF β inducible early growth responses gene (TIEG) and tryptophanyl-tRNA synthetase (WARS), were two out of 78 genes (2.6%) that are involved in cell proliferation were regulated by EGCG in the current study (Table 4, Supplemental Table 1). TIEG belongs to a family of transcription factors with anti-proliferative and apoptosis-inducing functions.^(27, 28) However, we found that this gene was down regulated by EGCG treatment (Supplement 1), whereas with VEGF stimulation, TIEG was upregulated by EGCG compared to the control (Supplement 2). Thus, the downregulation of TIEG represents the anti-VEGF function of EGCG and might be tissue-specific. WARS is an enzyme that catalyzes the first step of protein synthesis, and is reported to have antiangiogenesis function in addition to the catalyzing function.^(29, 30) It binds to intercellular junctions of endothelial cells to form a complex with VE-cadherin which then inhibits VEGF-induced ERK activation and endothelial cell migration.⁽³¹⁾ The up-regulation of WARS by EGCG and inhibition of angiogenesis may counteract down-regulation of TIEG as noted with EGCG treatment (Supplemental Table 2). In terms of

biological outcome, our finding is in accordance with the previous observations showing the suppressive effect of EGCG on the proliferation of different cell types. For examples, studies have shown EGCG inhibited the growth of human epidermoid carcinoma cells⁽³²⁾ hepatic stellate cells,⁽³³⁾ human bronchial epithelial 21BES cells⁽³⁴⁾ and human cervical cancer cells⁽³⁵⁾. One study reported that EGCG broadly decreased the expression of genes related with prostate cancer cells proliferation.⁽³⁶⁾ And another study reported EGCG modulated early-response genes in bronchial epithelial 21BES cells followed by activation of genes with a variety of cellular functions such as growth inhibition and the activation of apoptosis that are downstream targets of early-response genes.⁽³⁴⁾

Two pathways related to cell proliferation were modified by EGCG treatment in our study: Wnt signaling pathway and Id pathway. Wnts are a family of signaling proteins secreted from various cells, which have a major impact on embryonic development, tumor progression and stem cell differentiation.⁽³⁷⁾ (Please see the Wnt signaling pathway at: http://www.genmapp.org/HTML_MAPPs/Human/Hs_Contributed_20051123/cellular_process-GenMAPP/Hs_Wnt_signaling/Hs_Wnt_signaling.htm). Studies have shown that Wnt pathway is activated in endothelial cells *in vitro*. The activated endothelial cells express multiple ligands, receptors and secretory modulators, which may play roles in angiogenesis.⁽³⁸⁾ Wnt signaling also induces proliferation and survival of endothelial cells.^(39, 40) This pathway is triggered by wnt binding to its cell membrane receptors, which is composed of 10 transmembrane proteins.⁽⁴¹⁾ The signal is then transduced through several cascades of cytoplasmic proteins to nucleus via the translocation of the complex formed by β -catenin and LEF/TCF. The complex exerts transcriptional activity.⁽⁴²⁾ In the current study, 2 out of 77 genes in Wnt pathway were regulated by EGCG treatment. Dickkopf homolog 1 (Dkk1) is a soluble antagonist of wnt pathway and has a high affinity ligand to LRP5, which is a key molecule in Wnt pathway functioning as a co-receptor for Wnt binding to the cell membrane.⁽⁴³⁾ The induction of Dkk1 expression by EGCG in our study (Table 4 and Supplemental Table 1) is an indication of Wnt signaling pathway inhibition. Centromere protein E (CENPE) is a kinesin-like motor protein that accumulates in the G2 phase of the cell cycle. CENPE is proposed to be one of the motors responsible for mammalian chromosome movement and/or spindle elongation, and involved in Wnt signaling pathway. Its reduction by EGCG (Table 4 and Supplemental Table 1) further indicates the inhibition of cell proliferation by EGCG. In addition, inhibitors of differentiation (Id) or DNA binding proteins are important for cellular proliferation and differentiation in a variety of cell types through regulation of gene expression (please see Id pathway at this site: http://www.genmapp.org/HTML_MAPPs/Human/Hs_Contributed_20060824/cellular_process-GenMAPP/Hs_Id_NetPath_5/Hs_Id_NetPath_5.htm). Id-1 belongs to a group of helix-loop-helix proteins that lack DNA binding domain. Id-1 inhibits DNA binding activity of basic helix-loop-helix (bHLH) by forming non-functional heterodimers with it to inhibit cell differentiation and promote proliferation.⁽⁴⁴⁾ Studies have shown that Id-1 induces proliferation in prostate cancer cells, hepatocellular carcinoma cells and nasopharyngeal carcinoma cells.⁽⁴⁴⁾ Its down-regulation by EGCG (Table 4 and Supplemental Table 1) is likely to play a role in the inhibition of tumorigenesis and progression. Hairy and enhancer of split 1 (HES1) is another HLH-type of negative regulator of cell proliferation.^(45, 46) The down-regulation of this gene by EGCG (Table 4 and Supplemental Table 1) is probably results in the inhibition of proliferation. The inhibition of genes involved in cell proliferation by EGCG was also confirmed by the dose-dependent inhibition of HUVEC treated with EGCG or stimulated with VEGF following EGCG supplement (Figure 1). Our findings on the EGCG suppressive effect on vascular endothelial cells proliferation and those reported on cancer cells suggest that the overall anti-proliferative action of EGCG might be one important mechanism by which this polyphenols of green tea contributes to the prevention of angiogenesis and cancer progression.

We and others have shown that EGCG inhibits angiogenesis.^(12, 47) While endothelial cell proliferation is one of the stages of angiogenesis, other molecular mechanisms are also involved in this process. In the present study, we have identified relevant molecules involved in angiogenesis as affected by EGCG. GO term analysis discovered that EGCG has profound influence on intracellular microtubule components and activities (Tables 4 and 5), which may play a role in the inhibition of cell migration and angiogenesis as well. Further studies are needed to determine EGCG modulation at protein levels in conjunction with biological functions presented here: the inhibition of cell proliferation and angiogenesis.

The effects of EGCG on endothelial cells stimulated with VEGF—When EGCG-pretreated HUVECs were stimulated with VEGF, a more robust effect of EGCG on biological functions and signal transduction pathways was observed compared to the HUVECs treated with EGCG alone (Tables 5 and Supplemental Table 2). However, despite of EGCG inhibition of cell growth and angiogenesis (Figures 1 and 2), we did not see any change with EGCG pre-treatment in the most well known genes that are involved in the classic angiogenesis-signaling pathway such as PI3 kinase and dual specificity phosphatase (PTEN). These observations were in concurrence with the previous report demonstrating that EGCG had no effect on the expression of PI3 kinase and PTEN.⁽³⁶⁾ Our previous study also showed that EGCG inhibits angiogenesis by preventing phosphorylation of several enzymes involved in angiogenesis signal transduction pathways.⁽⁴⁸⁾ EGCG, through its direct binding to receptors and enzymes,^(49, 50) or through its metal chelating activity,⁽⁵¹⁾ may regulate some enzymes and cell surface receptors, which are dependent on divalent cation for their activation.^(8, 51) Furthermore, we have shown that EGCG inhibits angiogenesis through the disruption of VEGF-induced VE-cadherin/VEGF receptors/ β -catenin/PI3-kinase complex formation⁽⁴⁸⁾ in endothelial cells without affecting gene expressions of the enzymes. Therefore, the anti-angiogenesis effect of EGCG is mainly due to the inhibition of the activity of enzymes that are involved in signaling pathways that govern vessel formation and maturation.

When HUVECs were incubated with EGCG for 24hrs, then stimulated with VEGF for 30 min, there was no significant difference on gene expression compared to the cells that were stimulated with VEGF only. However, in comparison to EGCG treatment, 123 genes showed differential expressions. This differential effect can be attributed to the strong mitogenic effect of VEGF. VEGF has been shown to exert a dynamic modulatory effect on endothelial cell gene expressions associated with cell proliferation, migration, and angiogenesis.⁽¹⁷⁾

VEGF at the concentrations of 50 ng/mL or more has been used to investigate angiogenesis in cell culture systems.^(52, 53) In our previous studies we have also used 50 ng/mL of VEGF *in vitro* to determine its mechanism of action on angiogenesis.⁽⁴⁸⁾ To be consistent, we decided to use the same concentration of VEGF in the present study, which altered the expression of more than 350 genes in HUVECs within 30 min (Supplemental Table 3). This effect of VEGF is mediated through specific binding to its receptors; VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) on endothelial cells and downstream activating intracellular signaling pathways.⁽⁵⁴⁾

We have validated our findings by determining mRNA levels of several selected genes using real-time RT-PCR (Figure 3). Our PCR results have shown consistent and significant expression patterns of selected genes from Microarray analysis, which confirm our findings with Microarray analysis.

3. Summary

This study demonstrates the modulation of endothelial cell gene expression by EGCG when the cells were challenged with angiogenic growth factor VEGF. We have found that a combination of various cellular regulatory components is required for EGCG to exert its antiangiogenic effect. A selective number of genes modulated by EGCG were validated by real time PCR. It should be noted that *in vitro* levels of VEGF and EGCG applied in this study are comparable to those used by other investigators.^(35, 36, 55) The anti-angiogenic activity of EGCG on endothelial cells might be through inhibition of cell proliferation and cell migration. Future works are needed to elucidate the biological function of those specific genes by employing transgenic and molecular biology approaches. These EGCG-responsive genes may provide key insights for identifying the mechanisms of other polyphenolic compounds that might have cancer prevention properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This manuscript is based on work supported by the U.S. Department of Agriculture, under agreement No. 58-1950-9-001 and NCI grant #R03 CA094290-01. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture. We would also like to thank Stephanie Marco for her assistance in the preparation of this manuscript.

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Figure 1. The inhibitory effect of doses of EGCG with and without VEGF-stimulation on HUVEC proliferation

HUVEC were cultured in 6-well plate until 60% confluent. Cells were incubated with or without 5, 10 and 20 μ M EGCG in culture medium for 24 h, then stimulated with or without 50 ng/mL VEGF for 24 h. The cell number per well was determined as described in the method. Data are mean $\pm$ SEM of duplicates per treatment. * p <0.05 and ** p <0.001 compared to control. † p <0.05, ‡ p <0.001 compared to VEGF.

A **B**

Figure 2. The inhibitory effect on angiogenesis of HUVEC on Matrigel

HUVEC were cultured until 70% confluent. Cells were preincubated with or without EGCG 20 μ M for 24 h. Then the cells were seeded on growth factor reduced Matrigel coated 24-well plates and stimulated with or without 50 ng/mL VEGF for 48 h (see materials and methods).

(A) Tube formation by endothelial cells on Matrigel. (B) Quantification of total length and numbers of branches of formed tubes by HUVEC. Data mean $\pm$ SEM, n=3 per group. Three random fields in each well were selected to quantify the length and numbers of branches formed *in vitro* after 48 h. * p <0.05 compared with control cells, # p <0.05 compared with VEGF treated cells.

Figure 3. QPCR validation of selected differentially expressed genes by EGCG treatment
HUVECs were cultured in 100 mm dishes until 70% confluent. Cells were incubated with or without EGCG 20 μ M for 24 h. Total RNA was extracted for both microarray and real-time PCR analysis (see materials and methods). n=3 per treated group.

Table 1

Total numbers of genes that have been regulated by EGCG and VEGF

Treatment	Up-regulated		Down-regulated	
	*P 0.05	#Q 0.05	P 0.05	Q 0.05
EGCG vs Control	529	14	492	14
VEGF vs Control	710	291	638	51
EGCG + VEGF vs VEGF	372	0	325	0
EGCG + VEGF vs EGCG	425	116	257	7
Total	2036	421	1712	72

* P values were calculated based on Bayesian framework t-test.⁽¹⁹⁾

Q-value was estimated using Q-value software⁽²¹⁾ at the false discovery rate of 5%.

Table 2
GO Categories Over-Represented in Probe Sets Significant for VEGF vs. Control

GOID	GO Name*	Number Changed/Number Measured (% Changed)	Z Score	P value
Biological Process				
7250	activation of NF-kappaB-inducing kinase	2/7 (28.6%)	3.563	0.035
1525	angiogenesis	4/25 (16.0%)	3.870	0.001
6916	anti-apoptosis	10/52 (19.2%)	5.486	0.002
19722	calcium-mediated signaling	2/7 (28.6%)	3.563	0.026
7050	cell cycle arrest	7/42 (16.7%)	4.584	<0.001
1709	cell fate determination	2/5 (40%)	7.637	<0.001
7267	cell-cell signaling	17/92 (18.5%)	5.691	<0.001
6935	chemotaxis	9/37 (24.3%)	7.571	<0.001
30574	collagen catabolism	2/7 (28.6%)	3.563	0.022
7253	cytoplasmic sequestering of NF-kappaB	2/3 (66.7%)	5.886	0.003
6959	humoral immune response	2/13 (15.4%)	2.306	0.029
188	inactivation of MAPK activity	3/11 (27.3%)	4.236	0.004
50930	induction of positive chemotaxis	2/2 (100%)	7.345	0.001
6954	inflammatory response	6/61 (9.8%)	3.656	0.003
7229	integrin-mediated signaling pathway	4/26 (15.4%)	3.249	0.013
7259	JAK-STAT cascade	2/13 (15.4%)	2.871	0.034
30216	keratinocyte differentiation	2/3 (66.7%)	5.886	<0.001
7638	mechanosensory behavior	2/3 (66.7%)	5.886	0.003
7517	muscle development	4/49 (8.2%)	2.692	0.019
7422	peripheral nervous system development	2/3 (66.7%)	5.886	0.001
6470	protein amino acid dephosphorylation	7/74 (9.5%)	2.741	0.017
8277	regulation of G-protein coupled receptor protein signaling pathway	3/12 (25.0%)	4.001	0.007
9615	response to virus	3/21 (14.3%)	2.647	0.040
9611	response to wounding	2/8 (25.0%)	4.697	<0.001
1501	skeletal development	5/33 (15.2%)	2.732	0.013

GOID	GO Name*	Number Changed/Number Measured (% Changed)	Z Score	P value
6366	transcription from RNA polymerase II promoter	10/115 (8.7%)	4.890	<0.001
7179	transforming growth factor beta receptor signaling pathway	3/14 (21.4%)	2.871	0.031
1570	vasculogenesis	2/4 (50.0%)	5.002	0.011
Molecular Function				
8009	chemokine activity	6/11 (54.5%)	9.112	<0.001
5125	cytokine activity	6/31 (19.4%)	8.820	<0.001
3677	DNA binding	19/506 (3.8%)	5.322	<0.001
5355	glucose transporter activity	2/4 (50%)	5.002	0.007
8083	growth factor activity	11/55 (20.0%)	6.423	<0.001
5179	hormone activity	5/17 (29.4%)	5.346	<0.001
4879	ligand-dependent nuclear receptor activity	2/5 (40.0%)	4.308	0.002
17017	MAP kinase phosphatase activity	3/8 (37.5%)	5.171	<0.001
8195	phosphatidate phosphatase activity	2/3 (66.7%)	5.886	0.002
8243	plasminogen activator activity	2/5 (40.0%)	4.388	0.008
19901	protein kinase binding	3/11 (27.3%)	2.546	0.044
3707	steroid hormone receptor activity	5/24 (20.8%)	4.431	0.001
3714	transcription corepressor activity	9/58 (15.5%)	4.920	<0.001
Cellular Component				
5576	extracellular region	9/120 (7.5%)	5.111	<0.001
5615	extracellular space	16/103 (15.5%)	6.439	<0.001

* GO terms listed met three criteria:
 (1) Z score 1.96
 (2) Number genes with changed expression 2.
 (3) Unique GO term only.

Table 3

Pathway Analysis*

MAPP Name	Number Changed/Number Measured (% Changed)	Z Score	P value
EGCG vs Control			
Hs_Cholesterol_Biosynthesis	3/15 (20%)	8.946	<0.001
Hs_Id_NetPath_5	2/43 (4.7%)	3.117	0.039
Hs_Wnt_NetPath_8	2/77 (2.6%)	2.016	0.108
VEGF vs Control			
Hs_Hypertrophy_model	7/19 (36.8%)	5.230	<0.001
Hs_Adipogenesis	15/98 (15.3%)	3.431	0.002
Hs_Cytokines_and_Inflammatory_Response_Biocarta	4/16 (25%)	2.906	0.022
Hs_Apoptosis	10/73 (13.7%)	2.388	0.028
Hs_Nuclear_Receptors	5/27 (18.5)	2.438	0.032
EGCG vs EGCG+VEGF			
Hs_Hypertrophy_model	5/19 (26.3%)	6.265	<0.001
Hs_Cytokines_and_Inflammatory_Response_Biocarta	4/16 (25.0%)	5.424	0.001
Hs_Id_NetPath_5	4/43 (9.3%)	2.626	0.038
Hs_Delta-Notch_NetPath_3	5/65 (7.7%)	2.445	0.039
Hs_Blood_Clotting_Cascade	2/13 (15.4%)	2.771	0.047
Hs_Smooth_muscle_contraction	6/101 (5.9%)	1.978	0.054
Hs_Adipogenesis	6/98 (6.1%)	2.059	0.060
Hs_Prostaglandin_synthesis_regulation	2/20 (10%)	1.972	0.098

* Pathway listed met two criteria:

- (1) Z score ≥ 1.96
- (2) Number genes with changed expression ≥ 2 .

Table 4

GO Categories Over-Represented in Probe Sets Significant for EGCG vs. Control

GOID	GO Name*	Number Changed/Number Measured (% Changed)	Z Score	P value
Biological Process				
6695	cholesterol biosynthesis	3/17 (17.6%)	11.187	<0.001
7018	microtubule-based movement	2/40 (5.0%)	4.751	0.014
8285	negative regulation of cell proliferation	2/78 (2.6%)	3.221	0.036
Molecular Function				
3777	microtubule motor activity	2/64 (3.1%)	3.276	0.038
16491	oxidoreductase activity	2/33 (6.1%)	5.360	0.007
16853	isomerase activity	5/251 (2.0%)	3.566	0.004
Cellular Component				
5783	endoplasmic reticulum	4/194 (2.1%)	3.088	0.017
5624	membrane fraction	3/201 (1.5%)	3.332	0.017
5875	microtubule associated complex	2/27 (7.4%)	4.448	0.010

* GO terms listed met three criteria:

- (1) Z score 1.96
- (2) Number genes with changed expression 2.
- (3) Unique GO term only.

Table 5
GO Categories Over-Represented in Probe Sets Significant for EGCG+ VEGF vs. EGCG

GOID	GO Name*	Number Changed/Number Measured (% Changed)	Z Score	P value
Biological Process				
1525	angiogenesis	3/25 (12.0%)	4.275	0.007
6916	anti-apoptosis	5/52 (9.6%)	4.902	<0.001
6874	calcium ion homeostasis	2/5 (40.0%)	4.349	0.016
19722	calcium-mediated signaling	2/7 (28.6%)	6.448	0.002
7050	cell cycle arrest	3/42 (7.1%)	3.405	0.018
6928	cell motility	2/59 (3.4%)	3.749	0.006
7267	cell-cell signaling	12/92 (13.0%)	7.085	<0.001
6935	chemotaxis	7/37 (18.9%)	10.911	<0.001
7186	G-protein coupled receptor protein signaling pathway	4/88 (4.5%)	3.840	0.003
6959	humoral immune response	2/13 (15.4%)	4.092	0.003
50930	induction of positive chemotaxis	2/2 (100%)	12.459	<0.001
6954	inflammatory response	6/61 (9.8%)	7.760	<0.001
7229	integrin-mediated signaling pathway	2/26 (7.6%)	2.927	0.048
30216	keratinocyte differentiation	2/3 (66.7%)	10.108	0.001
7517	muscle development	2/49 (4.1%)	2.588	0.039
8285	negative regulation of cell proliferation	7/78 (9.0%)	6.054	<0.001
122	negative regulation of transcription from RNA polymerase II promoter	3/39 (7.7%)	3.525	0.016
7399	neurogenesis	8/107 (7.5%)	5.878	<0.001
9887	organogenesis	3/33 (9.1%)	8.144	<0.001
8284	positive regulation of cell proliferation	6/55 (10.9%)	5.996	0.001
8217	regulation of blood pressure	2/8 (25.0%)	5.992	0.006
8277	regulation of G-protein coupled receptor protein signaling pathway	2/12 (16.7%)	4.762	0.008
9618	response to pathogenic bacteria	2/7 (28.6%)	5.288	0.012
1501	skeletal development	3/33 (9.1%)	3.240	0.034
6366	transcription from RNA polymerase II promoter	4/115 (3.5%)	4.025	<0.001

GOID	GO Name*	Number Changed/Number Measured (% Changed)	Z Score	P value
Molecular Function				
8009	chemokine activity	6/11 (54.5%)	15.778	<0.001
5125	cytokine activity	3/31 (9.7%)	12.01	<0.001
3677	DNA binding	10/506 (2.0%)	5.311	<0.001
8083	growth factor activity	7/55 (12.7%)	7.904	<0.001
8201	heparin binding	3/29 (10.3%)	4.369	0.008
5179	hormone activity	2/17 (11.8%)	3.604	0.022
17017	MAP kinase phosphatase activity	3/8 (37.5%)	9.148	0.001
8243	plasminogen activator activity	2/5 (40.0%)	7.729	0.002
3707	steroid hormone receptor activity	3/24 (12.5%)	4.795	0.001
3714	transcription corepressor activity	5/58 (8.6%)	5.015	<0.001
Cellular Component				
5576	extracellular region	4/121 (3.3%)	6.595	<0.001
5615	extracellular space	11/103 (10.7%)	8.432	<0.001

* GO terms listed met three criteria:

- (1) Z score 1.96
- (2) Number genes with changed expression 2.
- (3) Unique GO term only.