Modulation of Gene Expression by (−)-Epigallocatechin Gallate in PC-9 Cells Using a cDNA Expression Array

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Green tea is the most effective cancer preventive beverage. In the light of this, the mechanisms of action of tea polyphenols were investigated on the molecular levels. We present here the effects of (−)-epigallocatechin gallate (EGCG) on expression of 588 genes in human lung cancer cell line PC-9 cells, using a human cancer cDNA expression array. The levels of gene expression in non-treated control cells, and cells treated with EGCG alone, with the tumor promoter okadaic acid alone, and with EGCG plus okadaic acid, were studied, and their expression levels were classified into down-regulation (under 0.5 fold) and up-regulation (over 2.0 fold) by comparing with the levels of control. Non-treated PC-9 cells expressed 163 genes out of 588, and EGCG-treated cells induced down-regulated expression of 12 genes and up-regulated expression of 4 other genes. From a comparison of gene expression in the cells treated with EGCG and in cells treated with EGCG plus okadaic acid, we found the following genes commonly affected by EGCG: down-regulation of four genes, NF-κB inducing kinase (NIK), death-associated protein kinase 1 (DAPK 1), rhoB and tyrosine–protein kinase (SKY); up-regulation of one gene, retinoic acid receptor α1. Among them, we think down-regulation of NIK gene expression is significant for cancer prevention, based on evidence that inhibition of NF-κB activation is a result of inhibition of NIK/IKK signalling complex.

Key words cancer chemoprevention; tea polyphenol; NF-κB inducing kinase; okadaic acid

MATERIALS AND METHODS

Materials EGCG was isolated from Japanese green tea leaves13); purity was 99.7%. Other tea polyphenols, (−)-epicatechin (EC) and (−)-epicatechin gallate (ECG) were purchased from Funakoshi Co., Ltd., Tokyo, Japan. Okadaic acid was isolated from a black sponge Halichondria okadai, as described previously.12) PC-9 cells were processed one of four ways: some (1.2×10⁵) were treated with 200 μM EGCG for 7 h; some (1.6×10⁵) were treated with 100 nm okadaic acid for 6 h; and some (2.0×10⁵) were pretreated with 200 μM EGCG for 1 h followed by further treatment with 100 nm okadaic acid for 6 h; and non-treated cells (2.0×10⁵) were used for control. Total RNA was isolated from the cells by ISOGEN reagent (Nip-
pon Gene, Tokyo, Japan) as reported\textsuperscript{[15]} and poly(A)\textsuperscript{+} RNA was further purified by Oligotex-dT30 mRNA purification kit (Takara Biomedicals, Tokyo, Japan).

cDNA Probe Synthesis from Poly(A)\textsuperscript{+}RNA The 1\mu g of poly(A)\textsuperscript{+} RNA was converted into \(^{32}\text{P}\)-labeled cDNA using [\(\alpha\text{-}\text{P}\)]dATP and MMLV reverse transcriptase in the Master Mix according to the manufacturer’s procedure (CLONTECH Laboratories, Inc., CA, U.S.A.).\textsuperscript{[16]} \(^{32}\text{P}\)-Labeled cDNA probe was purified through CHROMA SPIN-200 DEPC-H\textsubscript{2}O column.\textsuperscript{[16]}

Hybridizing cDNA Probe to the Atlas cDNA Expression Array \(^{32}\text{P}\)-Labeled cDNA probes of the control cells (6.1\times10\textsuperscript{6} dpm), cells treated with EGCG (6.1\times10\textsuperscript{6} dpm), with okadaic acid (4.4\times10\textsuperscript{6} dpm), and with EGCG plus okadaic acid (4.3\times10\textsuperscript{6} dpm) were first denatured and then hybridized to the cDNA expression array 18 h at 68\degree C according to the manufacturer’s procedure.\textsuperscript{[16]} Radioactivity of the array was counted using BAS 2000 Image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Atlas Human Cancer cDNA Expression Array Each membrane of the array can hybridize cDNA of 588 genes, which includes 6 major categories:\textsuperscript{[16]} A, genes encoding cell cycle regulators, growth regulators, and intermediate filament markers; B, genes for apoptosis, oncogenes and tumor suppressor genes; C, genes encoding DNA damage response, repair \& recombination, cell fate \& development and receptors; D, genes for cell adhesion \& motility, and angiogenesis; E, genes encoding invasion regulators, and cell–cell interactions; F, genes for growth factors and cytokines. Nine housekeeping genes were included in an additional category G, which includes 6 major categories:\textsuperscript{[16]} A, genes encoding cell membrane of the array can hybridize cDNA of 588 genes, B, genes for cell death \& survival, EGF receptor (EGFR), and EGFR-related genes; C, genes encoding tumor suppressor genes; D, genes for cell adhesion \& motility, and angiogenesis; G, GAPDH as a qualitative control. Each of the results was the mean\pm S.D. of triplicate or quatriplicate analyses of RT-PCR.

RESULTS AND DISCUSSION

Down-Regulated Gene Expression by EGCG Non-treated PC-9 cells expressed 163 genes among 588, and their expression pattern was first compared with that of cells treated with EGCG. Among the genes affected by EGCG, expression of 12 genes was down-regulated (under 0.5 fold) and that of 4 other genes was up-regulated (at least 2.0 fold).

The down-regulated genes are shown in Table 1, and they include: two protein kinase genes related to apoptosis; tyrosine–protein kinase; three genes related to rho family small GTPase and regulator; MAP kinase p38\(\gamma\) and CDC 25B/M-phase inducer phosphatase 2 genes related to cell cycle; envoplakin and synapse-associated protein 102 genes related to cell–cell interaction; dishevelled 1 gene; and epidermal growth factor receptor (EGFR) gene.

Thus, down-regulation by EGCG of NIK gene expression can lead to reduced activation of NF-\(\kappa\)B. The results correlate with previous evidence that EGCG inhibited growth of various cancer cell lines. The other protein kinase is death-associated protein kinase (DAPK, DAPK), which is a calcium/calmodulin-dependent serine/threonine kinase carrying ankyrin repeats and death domain. Levy-Strumpf and Kimchi previously reported that DAPK displays strong tumor suppressive activity, linking the control of apoptosis to metastasis,\textsuperscript{[17]} but the precise relationship between down-regulation by EGCG of DAPK kinase and tumor suppressive activity has not yet been clarified. In addition, EGCG induced down-regulation of EGFR gene expression mimicking the senescent cells, which demonstrates the unresponsiveness of EGFR to EGF by a strong interaction between EGFR and

Table 1. Genes Down-Regulated by EGCG

<table>
<thead>
<tr>
<th>Genes</th>
<th>Relative expression level(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>NF-(\kappa)B inducing kinase (NIK)</td>
<td>1.26</td>
</tr>
<tr>
<td>Death-associated protein kinase 1 (DAPK1)</td>
<td>1.50</td>
</tr>
<tr>
<td>CDC42 GTPase-activating protein</td>
<td>1.62</td>
</tr>
<tr>
<td>Envoplakin</td>
<td>1.31</td>
</tr>
<tr>
<td>MAP kinase p38(\gamma)</td>
<td>0.92</td>
</tr>
<tr>
<td>CDC25B/M-phase inducer phosphatase 2</td>
<td>1.51</td>
</tr>
<tr>
<td>Tyrosine–protein kinase (SKY)</td>
<td>2.09</td>
</tr>
<tr>
<td>Rho B</td>
<td>3.10</td>
</tr>
<tr>
<td>T-Lymphoma invasion and metastasis</td>
<td></td>
</tr>
<tr>
<td>inducing TIAM1</td>
<td>1.28</td>
</tr>
<tr>
<td>Dishevelled 1 (DVL1)</td>
<td>5.21</td>
</tr>
<tr>
<td>Synapse-associated protein 102 (SAP102)</td>
<td>1.95</td>
</tr>
<tr>
<td>Epidermal growth factor receptor (EGFR)</td>
<td>9.88</td>
</tr>
</tbody>
</table>

\(a\) Standard expression level of \(\beta\)-actin=10, based on radioactivity determined by BAS 2000 Image analyzer.
caveolins. To confirm the results, Fig. 1 shows the expression pattern of the genes (category B) for apoptosis, oncogenes and tumor suppressor genes.

**Up-Regulated Gene Expression by EGCG** Treatment with EGCG induced up-regulation of 4 genes in PC-9 cells (Table 2). Among them, expression of retinoblastoma binding protein (RBQ1) gene was elevated the most, 5.2 fold, whereas the other retinoblastoma binding protein, (RBP2) gene, was not expressed at all. Although the function of RBQ1 gene has not yet been elucidated, RBP2 stimulates transcription through binding to the Lim domain protein rhombolin-2 (RBTN-2). The other three genes whose expression was elevated by treatment with EGCG were: vascular endothelial growth factor (VEGF), retinoic acid receptor α1 (RAR-α1) and insulin-like growth factor-binding protein 3 (IGFBP 3) (Table 2). Of IGFBP 3 it is known that: it is identical to growth hormone-dependent insulin-like growth factor-binding protein by its number on GenBank; it is the principal IGFBP in circulation, and high levels of IGFBP 3 are associated with reduced lung cancer risk. The enhanced expression of phase II enzyme (GST), GSH-Px, catalase, NAD(P)H: quinone reductase, and gap junction proteins have been reported in cells treated with EGCG and green tea extract, and our results with the cDNA expression array confirmed some of these.

**Genes Commonly Affected by EGCG** Our initial results showed that okadaic acid induced up-regulated expression of 29 genes (over 2.0 fold) and down-regulated expression of 15 genes (under 0.5 fold) in PC-9 cells - a detailed description of the genes will be reported elsewhere. To these expression levels, pretreatment with EGCG further elevated expression of 2 genes and inhibited that of 21 genes; these results were then compared with those mentioned above obtained with EGCG alone. Ultimately, four genes were commonly down-regulated by EGCG: NIK and DAPK 1, rho B and SKY (Table 3). Since it is known that NIK activates the IKKα -IKKβ complex, leading to activation of NF-κB, down-regulated expression of the NIK gene seems to be involved in growth inhibition. One gene, retinoic acid receptor α1 gene, was commonly up-regulated by EGCG (Table 3).

**NIK Gene Expression in PC-9 Cells** In our concept of the mechanisms of action of green tea polyphenols, down-regulated expression of TNF-α is an essential process of cancer prevention. We therefore conducted semi-quantitative RT-PCR to confirm the expression level of the NIK gene as a model gene in PC-9 cells. Figure 2 shows that NIK gene expression was dose-dependently inhibited with EGCG (10—200 μM), and that GAPDH gene expression was unchanged. NIK gene expression in the cDNA expression array (Table 1) was down-regulated by 0.2 fold in cells treated with 200 μM EGCG, and its expression by semi-quantitative RT-PCR was...
down by 0.39 fold with the same concentration of EGCG (Fig. 2). Clearly, the results with RT-PCR are consistent with the results of the cDNA expression array. The down-regulated level of NIK gene expression by EGCG correlated well with the level of inhibition of NF-κB activation and growth inhibition of PC-9 cells. We previously reported that other tea polyphenols, such as (-)-epicatechin gallate (ECG) and (-)-epigallocatechin (EGC), are biologically active compounds, as is EGCG, whereas (-)-epicatechin (EC) is inactive.\(^1,11\) In addition to EGCG, we therefore studied the effects of ECG and EC on NIK gene expression in PC-9 cells by RT-PCR: Figure 2 shows that ECG inhibited it dose-dependently and EC did not. Furthermore, Plummer et al. recently reported that the cancer chemopreventive agent curcumin suppresses cyclo-oxygenase 2 expression in human colon epithelial cells by inhibiting the NIK-IκB kinase activation of NF-κB.\(^2\) Since EGCG also inhibits activation of NF-κB, EGCG apparently inhibits IκB phosphorylation by down-regulating the NIK/IκB signalling complex.

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REFERENCES AND NOTES

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