(-)-Epigallocatechin-3-Gallate (EGCG) Increases the Viability of Serum-Starved A549 Cells Through Its Effect on Akt

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Abstract: The effect of epigallocatechin gallate (EGCG) on cell survival was studied by using serum-starved A549 non-small cell lung carcinoma (NSCLC) cells. A MTT assay showed that EGCG significantly increased the viability of serum-starved A549 cells compared to the control cells, though EGCG at high concentration (∼300 µM) had no protective effect against serum withdrawal-induced cell apoptosis. Western blots showed increased immunoreactivity for phospho-Akt and phospho-GSK3β in EGCG-treated cells. To determine the mechanism for Akt phosphorylation, cells were pretreated with various kinase inhibitors before exposure to EGCG. Only LY294002 inhibited Akt activation induced by EGCG, implying that EGCG-induced Akt activation is PI3K dependent. Both phospho-Raf-1 and Raf-1 proteins were significantly decreased, whereas B-raf expression was not altered. This suggests that the Raf kinases have no role in the increased cell survival caused by EGCG. This study has shown that EGCG protects A549 cells from apoptosis induced by serum deprivation via Akt activation and this protective effect may limit the clinical use of EGCG in treating and preventing NSCLC.

Keywords: Green Tea; EGCG; Akt; Raf-1; A549; Cell Survival.

Introduction

Green tea, made from the dried leaves of the plant Camellia sinensis, is one of the most widely consumed beverages in the world. It is known to have beneficial health effects, such
as anti-inflammatory, anti-atherogenic, and anti-cancer effects (Miura et al., 2001; Suganuma et al., 1999; Mukhtar et al., 1994; Kumar et al., 2007; Ganguly et al., 2005). Studies have focused on the cancer chemopreventive action of green tea; the growth inhibitory effects of tea against carcinogenesis have been demonstrated in many epidemiological studies (Ohno et al., 1995; Mendilaharsu et al., 1998; Imai et al., 1997). The biological activity of green tea in tumor inhibition is also supported by many experimental studies by using in vitro and animal models (Yang et al., 2002; Wang et al., 1992; Sartippour et al., 2001). Although a large number of epidemiological and experimental studies have suggested the growth suppressive action of green tea on a variety of cancers, including stomach, colon, and breast cancers (Suganuma et al., 1999; Mukhtar et al., 1994; Kumar et al., 2007; Sartippour et al., 2001; Katiyar and Mukhtar, 1997), other studies have demonstrated that it has no inhibitory activity on tumors, and some even suggest green tea promoted tumor growth (Zheng et al., 1996; Tewes et al., 1990).

The association between tea consumption and lung cancer risk is not clear. While a few studies have found reduced risk of lung cancer development with the tea, some studies have observed no meaningful association between the two factors (Suganuma et al., 1999; Ohno et al., 1995; Mendilaharsu et al., 1998; Imai et al., 1997; Yang et al., 2002; Zheng et al., 1996; Goldbohm et al., 1996). Tewes et al. (1990) even reported an increased risk of developing lung cancer from green tea consumption. To provide insight into the inconsistency association between tea consumption and the risk of lung cancer, it is necessary to examine the effects and the mechanisms of action of green tea on lung cancer cells.

Lung cancer is the predominant cause of cancer mortality in both men and women. Non-small cell lung carcinoma (NSCLC) accounts for more than 85% of all lung cancers and carries a poor prognosis, with a 5-year survival rate (from the time of diagnosis) rarely exceeding 15%. NSCLC demonstrates high resistance to various kinds of chemotherapeutic agents and this resistance limits the efficacy of numerous agents in many NSCLC patients (Hovelmann et al., 2004; Cheng et al., 2000). A study has reported that human lung adenocarcinoma A549 cells, which belong to NSCLC, are markedly resistant to apoptosis induced by (-)-epigallocatechin-3-gallate (EGCG), the most abundant and potent polyphenolic constituent in green tea (Kweon et al., 2006). Elucidation of the molecular mechanisms leading to this EGCG resistance can be used to extend the clinical usefulness of EGCG and green tea in lung cancers. The survival rate of lung cancer patients can be increased by approaches that overcome the main mechanisms of this resistance.

The phosphoinositide 3-kinase (PI3K)/Akt pathway represents an important signal transduction pathway that triggers a cascade of responses, from cell growth and proliferation to cell survival, which drives tumor progression (Vivanco and Sawyers, 2002). It is generally considered to work in parallel with the RAS/Raf/MAPK pathway, which regulates cell proliferation. Raf-1 is a kinase activating a mitogen-activated protein kinase (MAPK) cascade; this pathway is associated with the promotion of survival genes and inhibition of apoptotic signals, leading to cell survival (Cleveland et al., 1994; Erhardt et al., 1999; Xia et al., 1995). We considered that these pathways might be involved in EGCG-mediated effects on
A549 cells. In this study, the effect of EGCG on the survival of NSCLC cells was evaluated. In addition, the molecular mechanisms underlying the effects of EGCG were investigated by focusing on PI3K/Akt and Raf/MAPK signaling pathways.

**Materials and Methods**

**Reagents**

EGCG was obtained from Sigma (St. Louis, MO, USA), dissolved in distilled water at a concentration of 100 mM, and stored at −80°C as a stock solution. PPP (insulin growth factor receptor inhibitor), PP2 (Src inhibitor), PD168393 (epidermal growth factor receptor inhibitor), and LY294002 (PI3K inhibitor) were purchased from Calbiochem (San Diego, CA, USA). DMPQ (platelet-derived growth factor receptor inhibitor) was obtained from TOCRIS (Ellisville, MO, USA). All the inhibitors were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration did not exceed 0.1% throughout the study. Primary antibodies against Akt, phospho-Akt (Ser 473), 3-phosphoinositide dependent kinase 1 (PDK1), phospho-PDK1, glycogen synthase kinase 3β (GSK 3β), phospho-GSK 3β (Ser 9), Phosphatase and TENsin homolog (PTEN), phospho-PTEN, p44/42 MAPK, and phospho-p44/42 MAPK were obtained from Cell Signaling (Beverly, MA, USA). Anti-Raf-1, anti-phospho-Raf-1, anti-B-raf, and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals such as aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma.

**Cell Culture**

Human NSCLC A549 cells were maintained in RPMI 1640 media containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (GIBCO, Grand Island, NY, USA). Cultures were kept at 37°C in a humidified atmosphere incubator with 5% CO₂.

**Cell Viability Analysis**

Approximately 1 × 10⁴ A549 cells were plated in each well in 96-well plates. After overnight incubation, media was changed to serum-free media. The next day, cells were pretreated with EGCG (0–600 µM) for 1 hour, 3 hours, or 6 hours. Then, cell growth continued in EGCG-free and serum-free media. After 1 day, the media was removed, and a total of 50 µl of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) was added at a concentration of 2 mg/ml in each well for 2 hours. The solution was removed from each well, and then 200 µl of DMSO was added. Optical density was evaluated at 540 nm by using an ELISA plate reader. The viability of EGCG pretreated cells was expressed as a percentage compared to non-EGCG treated control cells. The assay was performed in triplicate.
Western Blot Analysis

Briefly, cells were washed twice with ice-cold PBS, lysed with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% sodium dodecyl sulfate (SDS), 100 μg/ml PMSF, 50 μl/ml aprotinin, 1% Nonidet P-40, 100 mM sodium fluoride, 0.5% sodium deoxycholate, 0.5 mM EDTA, and 0.1 mM EGTA). Protein concentrations of the cell lysates were determined by Bradford assay (Bio-Rad, Richmond, CA, USA). An equal amount (50 μg) of protein from each sample was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). The membranes were blocked with 5% skim milk, and then incubated overnight with the appropriate primary antibody. The membranes were washed with Tris-buffered saline containing 0.05% Tween-20, and then processed by using the appropriate secondary antibody. Bound secondary antibody was detected by using SuperSignal West-Femto chemiluminescence substrate (Pierce, Madison, WI, USA).

Results

EGCG Protects A549 Cells from Serum Deprivation-Induced Death

As shown in Fig. 1, pretreatment with EGCG for 1 hour or 3 hours significantly increased cell viability and the increase was dose-dependent. The cell viability peaked when cells were pretreated with 200 μM of EGCG for 1 hour. When cells were exposed to EGCG for 3 hours, 100 μM EGCG was most efficient in increasing cell viability. Pretreatment with equal or greater than 300 μM EGCG did not show any protective effect against cell death. When cells were pretreated with EGCG for 6 hours, even 10 μM EGCG would protect cells against serum withdrawal. This protection was weakened as EGCG concentration was increased above 50 μM, and was absent at concentration of 200 μM. Furthermore, higher concentrations of EGCG seemed to induce cell death and decrease cell viability. These findings suggest that the protection by EGCG is most effective when cells are exposed to EGCG for short-period at 100 or 200 μM concentrations, and that higher concentrations or prolonged exposure to EGCG does not efficiently protect cells from a death signal. For subsequent experiments, 3 hours pretreatment and 100 μM EGCG concentration were used.

EGCG Increases Akt Kinase Activity

To understand the mechanism of the protective function of EGCG against apoptosis, the effect of EGCG on Akt signaling was examined. Assessment of the phosphorylation status of Akt following the addition of EGCG for 3 hours was performed by using antibodies that specifically recognize the serine (473) of Akt, where the phosphorylation status is indicative of its activation. The expression level of active phosphorylated Akt was significantly increased by EGCG treatment, though there was no obvious change in the expression level of total Akt (Fig. 2A).
Figure 1. Viability of A549 cells treated with EGCG. Cells were serum starved overnight and were incubated with 0, 10, 25, 50, 100, 200, 300, 400, 500, and 600 µM of EGCG respectively for 1 hour, 3 hours, or 6 hours. The next day, the number of surviving cells was measured and compared to the control group.
Figure 2. Effects of EGCG on Akt activity in A549 cells. Cells were serum starved overnight and treated with various concentrations of EGCG for 3 hours. Cell lysates (50 µg) were prepared and subjected to Western blot analysis with antibodies against phospho-Akt (A) or phospho-GSK 3β (B), as indicated.

GSK 3β is a crucial mediator located downstream from Akt signaling and is phosphorylated by active Akt. As shown in Fig. 2B, EGCG increased the phosphorylation of GSK 3β, showing that Akt kinase was actively signaling in this way.

**Effect of Kinase Inhibitors on EGCG-Induced Akt Activation**

To investigate how EGCG activates Akt, the status of PTEN and PDK1, which are upstream mediators of Akt signaling, was examined. EGCG increased dephosphorylated forms of PTEN and PDK1, forms which suppress phosphorylation of Akt (Fig. 3). This finding contradicts the idea of increased phosphorylation of Akt by EGCG. To find an upstream activator involved in EGCG-mediated Akt activation, cells were treated with various kinase inhibitors for 1 hour, then 100 µM EGCG was applied to the cells for 3 hours. As shown in Fig. 4, EGCG-induced Akt activation was completely blocked by a PI3K inhibitor, whereas none of the other kinase inhibitors had inhibitory effects on Akt activation.

**EGCG Decreases Raf-1 Protein Expression**

Another important pathway that is essential for cell survival is the Raf/MAPK pathway. To investigate whether Raf is involved in the increased cell viability caused by EGCG, the immunoreactivities of Raf kinases were checked. EGCG significantly decreased not only phospho-Raf-1 but also the total amount of Raf-1 protein, while B-raf expression was not altered. Although Raf-1 was not activated, p44/42 MAPK, its downstream target, was increasingly phosphorylated by EGCG (Fig. 5).
Figure 3. Change of phosphorylation pattern in PDK1 and PTEN after exposure of A549 cells to EGCG. Cells were serum starved overnight and treated with EGCG for 3 hours. Cell lysates (50 µg) were prepared and subjected to Western blot analysis.

Figure 4. Effects of kinase inhibitors on Akt phosphorylation in A549 cells. Serum-starved cells were pretreated with various kinase inhibitors, as indicated, for 1 hour prior to EGCG treatment. Then, cells were treated with 100 µM of EGCG for 3 hours and lysed. Lysates were analyzed using Western blotting. PPP, 2 nM Picropodophyllin, IGF1R inhibitor; PP2, 10 nM PP2, Src inhibitor; DMPQ, 200 nM DMPQ, PDGFR inhibitor; PD, 1 µM PD168393, EGFR inhibitor; LY, 20 µM LY 294002, PI3K inhibitor.

Discussion

A large number of studies have found that EGCG induces apoptosis or inhibits cell proliferation in lung, breast, and colon cancers (Kumar et al., 2007; Ganguly et al., 2005;
Figure 5. Activation of Raf kinases and p44/42 MAPK in A549 cells treated with EGCG for 3 hours. Cell lysates (50 µg) were prepared and subjected to Western blot analysis using the appropriate antibodies.

Sartippour et al., 2001). However, a few studies have shown inconsistent results. Koh et al. (2003) reported that EGCG protects cells against stress-induced apoptosis. Chung et al. (2003) have shown that EGCG induces cell proliferation and promotes keratinocyte survival. This differential sensitivity to EGCG may be due to a cell type-specific response. Ahmad et al. (1997) have suggested that EGCG triggers apoptosis only in cancer cells and not in normal cells. In the present study, EGCG did not induce apoptosis or suppress cell growth, but rather protected A549 cells against stress-induced apoptosis, despite the fact that A549 cells are derived from lung adenocarcinoma. However, prolonged exposure at high concentrations of EGCG decreased cell survival and suppressed cell growth (Fig. 1). Thus, it seems that cell responses to EGCG are more dependent on the concentration and exposure time rather than on cell types.

It is well known that Akt activation is essential for cell survival and provides a protective signal which blocks entry into apoptosis when cells are exposed to apoptotic stimuli such as serum withdrawal and matrix detachment (Vivanco and Sawyers, 2002; Dudek et al., 1997; Osaki et al., 2004). As mentioned above, EGCG was observed to increase the apoptotic threshold or delay the time course of onset of apoptotic pathways in serum-starved cells (Fig. 1). It was hypothesized that the resistance to apoptosis from EGCG might be a result of Akt activation. As expected, EGCG significantly increased phosphorylation of Akt at a concentration of 50 µM EGCG (Fig. 2), which coincided with the increased cell viability measured in the MTT assay. This differs from Akt inactivation in carcinoma cells and downregulation of phospho-Akt in bladder cancer cells (Sah et al., 2004; Qin et al., 2007).
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Interestingly, the downregulations in the previous reports were observed after 24 hours of EGCG treatment, whereas a shorter period of exposure to EGCG induced Akt activation in endothelial cells and COS7 cells (Wu et al., 2006; Kim et al., 2007). In this study, the increased Akt phosphorylation was observed after exposing A549 cells to EGCG for 3 hours. Overall, EGCG activates Akt at early time points, and this Akt activation might be a major contributor in increased cell survival rate due to EGCG.

Once it was activated, Akt exerts anti-apoptotic effects through phosphorylation of a substrate such as Bad or GSK 3, which directly regulate the apoptotic machinery. Studies have shown that activated Akt can influence the onset of apoptosis by phosphorylation of GSK 3β (Cantley, 2002; Cross et al., 1995). In this study, phosphorylation of GSK 3β was increased, implying that EGCG affects downstream signaling through the Akt pathway. Thus, the authors suggest that EGCG reduces apoptosis and enhances cell survival rate involving increased Akt activity.

The molecular mechanisms for Akt activation involve PTEN phosphorylation (inactivation) and/or PDK phosphorylation (activation), which can contribute directly to Akt activation (Baker, 2007; Wang and Brattain, 2006). The data from this study showed that EGCG treatment resulted in a significant and dose-dependent inhibition of PDK1 and PTEN phosphorylation (Fig. 3). This suggests that Akt activation by EGCG in A549 cells occurs neither through PDK1 activation nor through PTEN inactivation.

Akt is phosphorylated by phosphatidylinositol 3,4,5-triphosphate (PIP3), which is generated by active PI3K; the PI3K is activated by numerous receptor tyrosine kinases, such as epidermal growth factor receptor, platelet-derived growth factor receptor, and insulin growth factor receptor (Vivanco and Sawyers, 2002). In addition to activation by tyrosine kinases, PI3K/Akt can also be activated by the Src family of kinases. To investigate which pathway is used to activate Akt, inhibitors of tyrosine kinases and Src inhibitor were applied to A549 cells before EGCG treatment. Interestingly, no inhibitor except LY294002, a PI3K inhibitor, blocked EGCG-induced Akt activation. Only LY294002 completely eliminated Akt phosphorylation (Fig. 4). Although the precise mechanism by which EGCG protects cells against stress-induced apoptosis remains incompletely characterized, the present data suggest that the effects of EGCG on Akt activation are dependent on PI3K. Further study is needed to define specific signaling pathways that contribute to PI3K/Akt activation.

Several studies have suggested that Ras and its downstream effectors Raf-MEK-MAPK are critical mediators of cell survival in a number of different cell types (Xia et al., 1995; Le Gall et al., 2000; Garnett and Marais, 2004). Increased cell viability in this study could be due to Raf activation. However, it seems that Raf kinases do not play a role in the increased viability of A549 cells caused by EGCG, since both activity and protein expression of Raf-1 were decreased in the present study (Fig. 5A). Chung et al. (2001) observed that EGCG had no effect on the protein levels of Raf-1, while theaflavin-3,3′-digallate (TFdiG), a major polyphenol in black tea, effectively decreased total Raf-1 protein levels through lysosomal degradation. Taken together, it is clear that Raf kinases tend to be decreased by catechins including EGCG. Though most studies on the relation between EGCG and p42/44 MAPK have reported an inhibitory action of EGCG on MAPK activity, the present study showed an increased MAPK activity from EGCG treatment. It is difficult to explain the increased
MAPK activity, since Raf-1 kinase, an upstream activator of MAPK, was decreased. Further study is needed to define the effect of EGCG on the Raf/MAPK cascade.

In conclusion, the present study showed that the PI3K/Akt pathway is a major pathway mediating the effect of EGCG on improved survival rate in A549 cells.

References


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