GREEN TEA POLYPHENOL EGCG SIGNALLING THROUGH THE 67-kDa LAMININ RECEPTOR

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Summary

(–)-Epigallocatechin-3-gallate (EGCG), the principal polyphenol in green tea, has been shown to be a potent chemopreventive agent. Recently 67-kDa laminin receptor (67LR) has been identified as a cell surface EGCG receptor. However, the cell signalling pathway mediated after the binding of EGCG to 67LR is not entirely understood. Here we identify, using the genetic suppressor element approach, eukaryotic translation elongation factor 1A (eEF1A) as a key molecule responsible for mediating the EGCG signalling through 67LR. eEF1A has been known as not only an important component of the eukaryotic translation apparatus but also a multifunctional protein that is involved in a large number of cellular processes. Further, we found that through both eEF1A and 67LR, EGCG induces the dephosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) at Thr-696 and activates myosin phosphatase. Silencing of 67LR, eEF1A or MYPT1 in tumor cells results in abrogation of EGCG-induced tumor growth inhibition *in vivo*, supporting our conclusion that they serve as mediators for EGCG signalling.

Keywords

EGCG; 67LR; eEF1A; MYPT1; Cancer prevention

Introduction

Among the green tea constituents, (–)-epigallocatechin-3-gallate (EGCG) is the most abundant and most active constituent in inhibiting experimental carcinogenesis and related reactions[1]. Although many mechanisms for the anticancer activities of EGCG have been proposed based mainly on studies in cell lines, it is still not clear which EGCG-induced molecular events are responsible for its cancer-preventive activity *in vivo*[2].

Recently we have identified 67-kDa laminin receptor (67LR) as a cell surface EGCG receptor that mediates the anticancer action of EGCG[3]. In this study, we tried to illuminate the cell signalling pathway mediated after the binding of EGCG to 67LR and its biologic and physiologic significance for the cancer-preventive activity of EGCG *in vivo*.

Results and Discussion

We attempted to search for the mediators of EGCG-induced cell growth inhibition in B16 mouse melanoma cells using a targeted genetic screen with a genetic suppressor element (GSE) complementary DNA library which was prepared from a mouse embryo (Fig. 1). We expected that inactivation of genes essential for EGCG-induced cell growth inhibition by GSE sequences would allow cells to escape growth inhibition, continue cell growth and eventually form cell colonies even in the presence of EGCG. By using this screen, we isolated multiple GSE sequences and individually tested their ability to confer EGCG-resistance to B16 cells. The sequence for the most effective GSE corresponded to the N terminus of eukaryotic translation elongation factor 1A (eEF1A), a multifunctional protein that was originally identified as a cofactor for polypeptide elongation.

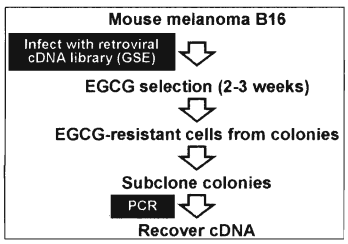

Figure 1 A flow chart describing a genetic screen for regulators of EGCG-induced cell growth inhibition. GSE, genetic suppressor element.

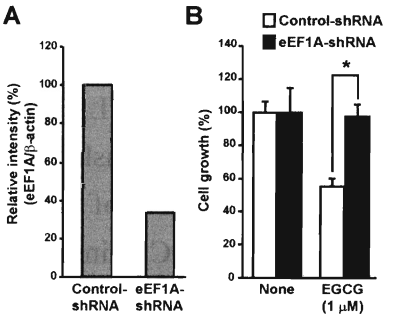

Figure 2 Effect of eEF1A knockdown on EGCG-induced cell growth inhibition in B16 cells. A, eEF1A knockdown in B16 cells stably transfected with the eEF1A-shRNA expression vector was confirmed by Western blot analysis. **B**, Cells were treated with the 1 μM EGCG for 96 h and cell number was assessed. The results are shown as relative cell number to untreated control and the data presented are the means ± s.d. (n=3) (asterisk indicates P<0.01).

To investigate the role of eEF1A in EGCG-induced cell growth inhibition, we used stable RNA interference (RNAi) to silence eEF1A expression in B16 cells. Remarkably, silencing of eEF1A attenuated the inhibitory effect of 1 μM EGCG on the cell growth (Fig. 2A, B). This concentration is similar to the amount of EGCG found in human plasma after drinking more than two or three cups of green tea[4]. Based on these considerations, the activities observed at 1 μM EGCG is relevant to the *in vivo* situations. Given this, we investigated the effect of oral administration of EGCG on subcutaneous tumor growth in C57BL/6N mice challenged with eEF1A-ablated B16 cells. Tumor growth was significantly retarded in EGCG-administered mice implanted with the B16 cells harbouring a control-short hairpin RNA (shRNA), whereas tumor growth was not affected by EGCG in the mice implanted with eEF1A-ablated B16 cells (Fig. 3), indicating that eEF1A is involved in EGCG-induced cancer prevention.

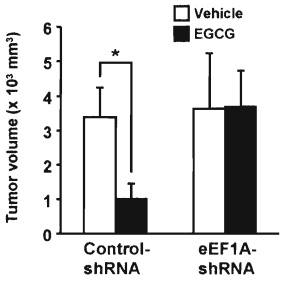

Figure 3 Effect of eEF1A knockdown in tumor cells on EGCG-induced tumor growth inhibition in murine tumor model. C57BL/6N mice were subcutaneously inoculated with B16 cells stably transfected with the control-shRNA or the eEF1A-shRNA expression vector. Peroral administration of 0.1% EGCG was started 1 day before the cell inoculation. Tumor sizes were determined 22 days after cell inoculation via caliper measurements. Data are represented as the mean ± s.e.m of 6-7 mice. Data were analyzed by Mann-Whitney U test.

Previously we reported that EGCG-induced cell growth inhibition may result from the reduction of the phosphorylation of myosin regulatory light chain (MRLC) at Thr-18/Ser-19, which controls the activity of myosin II, through 67LR in HeLa cells[5]. The MRLC phosphorylation is regulated by two classes of enzymes, MRLC kinases and myosin phosphatase. The activity of myosin phosphatase is known to be regulated by phosphorylation of its targeting subunit MYPT1 and two major sites, Thr-696 and Thr-853, have been extensively investigated and identified as an inhibitory site. We tested the effect of EGCG on the phosphorylation of MYPT1 at Thr-696 and Thr-853. Intriguingly, while the phosphorylation level at Thr-853 was unaffected by EGCG, EGCG induced the dephosphorylation of MYPT1 at Thr-696 in a dose-dependent manner (Fig. 4A). Further, this effect correlated with EGCG-induced reduction of the MRLC phosphorylation (Fig. 4B), suggesting EGCG activates myosin phosphatase by reducing the MYPT1 phosphorylation level at Thr-696.

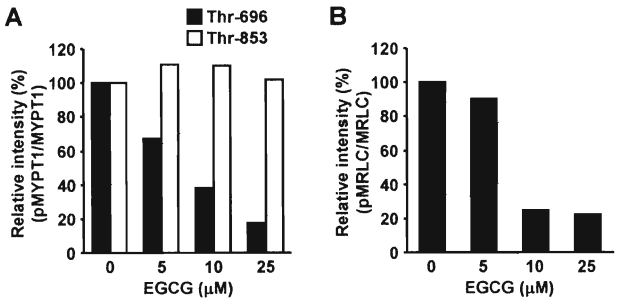

Figure 4 EGCG reduces the phosphorylation of MYPT1 at Thr-696. A, B, MYPT1 phosphorylation (Thr-696 and Thr-853) (**A**) and MRLC phosphorylation (Thr-18/Ser-19) (**B**) in HeLa cells exposed to the indicated concentrations of EGCG for 10 min were analyzed by Western blot analysis. Band intensities were quantified using NIH Image-J software.

Next we investigated whether EGCG-induced dephosphorylation of MYPT1 at Thr-696 is relevant to cancer-preventive action of EGCG *in vivo*. In B16 cells, physiological concentrations of EGCG reduced the MYPT1 phosphorylation at Thr-696 and the MRLC phosphorylation (data not shown). We confirmed that silencing of MYPT1 by stable RNAi in B16 cells and attenuation of the inhibitory effect of 1 μM EGCG on cell growth in MYPT1-ablated B16 cells *in vitro* (data not shown). We tested the effect of oral administration of EGCG on subcutaneous tumor growth in C57BL/6N mice challenged with MYPT1-ablated B16 cells. Tumor growth was significantly retarded in EGCG-administered mice implanted with the B16 cells harbouring a control-shRNA, whereas tumor growth was not affected by EGCG in the mice implanted with MYPT1-ablated B16 cells (data not shown), suggesting that MYPT1 is indispensable for EGCG-induced cancer prevention.

It has been reported that eEF1A binds to the ankyrin repeat of MYPT1. It is tempting to speculate that both 67LR and eEF1A are upstream signalling components responsible for EGCG-induced dephosphorylation of MYPT1 at Thr-696. To test this hypothesis, we used stable RNAi to silence the expression of 67LR or eEF1A in B16 cells. We confirmed specifical silencing of each target protein by stable RNAi in B16 cells and attenuation of the inhibitory effect of EGCG on cell growth in these cells

(data not shown). In both 67LR-ablated B16 cells and eEF1A-ablated B16 cells, the inhibitory effect of EGCG on both the phosphorylation of MYPT1 at Thr-696 and the phosphorylation of MRLC was attenuated (Fig. 5). These results suggest that MYPT1 is involved in downstream EGCG signalling from both 67LR and eEF1A.

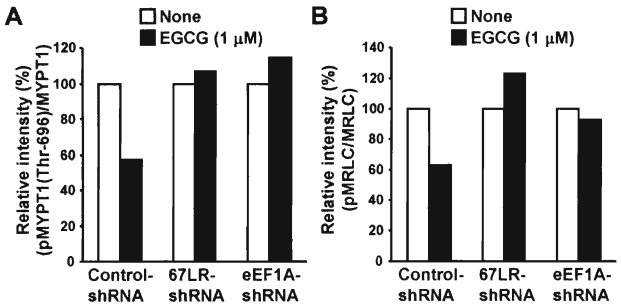

Figure 5 67LR and eEF1A mediate EGCG-induced reduction of the MYPT1 phosphorylation at Thr-696. A, **B**, MYPT1 phosphorylation at Thr-696 (**A**) and MRLC phosphorylation (Thr-18/Ser-19) (**B**) in B16 cells exposed to 1 μM EGCG for 20 min were analyzed by Western blot analysis. Band intensities were quantified using NIH Image-J software.

We recently reported that EGCG exerts its effects through its interaction with 67LR and others showed that RNAi-mediated silencing of 67LR resulted in abrogation of EGCG-induced apoptosis in myeloma cells. However, there is no validation of its implication in EGCG-induced cancer prevention *in vivo*. We investigated the effect of oral administration of EGCG on subcutaneous tumor growth in C57BL/6N mice challenged with 67LR-ablated B16 cells. Tumor growth was significantly retarded in EGCG-administered mice implanted with the B16 cells harbouring a control-shRNA, whereas tumor growth was not affected by EGCG in the mice implanted with 67LR-ablated B16 cells (data not shown), suggesting that 67LR functions as EGCG receptor not only *in vitro* but also *in vivo*.

Acknowledgements

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