Phosphodiesterase V selective inhibitor enhances pro-apoptotic effect of EGCG by enforcing EGCG signaling pathway through the 67 LR

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Summary

Epigallocatechin-3-gallate (EGCG) is a major polyphenol in green tea. We previously reported that biological activities of EGCG are depended on the 67-kDa laminin receptor (67LR), and this receptor has been previously reported to be abnormally elevated in multiple myeloma cells. In this study, we found that phosphodiesterase V selective inhibitor significantly enhanced pro-apoptotic effect of EGCG.

Introduction

In recent years, chemopreventive and chemotherapeutic effects of EGCG has been reported in different malignancies. EGCG selectively inhibits cell growth and induces apoptosis in cancer cells without adversely affecting normal cells.

We have previously identified the 67 kDa laminin receptor (67LR) as a cell-surface receptor that mediates the anticancer action of EGCG (Tachibana, *et al*. 2004). Recent studies revealed that 67LR is significantly elevated in both MM cells cells relative to normal peripheral blood mononuclear cells (PBMCs) and 67LR plays an important role in mediating EGCG activity in MM (Shammas, *et al*. 2006) while sparing PBMCs. These reports demonstrated that selective toxicity of EGCG and provide the rationale for its clinical evaluation. However the concentrations of EGCG that are required to elicit the anticancer effects are much higher than the peak plasma concentration observed in clinical trial (Shanafelt, *et al*. 2009). To enhance the anticancer effects of EGCG at a reasonable concentration in therapeutic, we investigated drugs which enhance EGCG/67LR signal pathway and sensitized malignant cells to EGCG without affecting normal cells.

Materials and methods

U266 human multiple myeloma cells were initially plated at a density of $5x10^4$ cells/well (3 wells/group) in 24 well plate and exposed to 5 μM Vardenafil a phosphodiesterase V (PDE V) selective inhibitor for 3 h before cells were treated with EGCG for 96 h. Apoptotic MM cells were detected using the Annexin V-Alexa Fluor 488 (Invitrogen,Carlsbad, CA). Cells (10^6 cells/mL) were mixed with Annexin V−Alexa Fluor 488 and media-binding reagent and incubated in the dark for 15 minutes at room temperature. Cells were then centrifuged and medium was replaced with binding buffer. A portion of cell suspension was placed onto a glass slide, covered with a coverslip, and viewed immediately using a fluorescence microscope equipped with green filter. For flow cytometory analysis, cells were double stained with Annexin V-Alexa Fluor 488 (Invitrogen,Carlsbad, CA) and propidium iodide (PI) at 2 μg/mL (Sigma). Percentages of annexin-V–positive cells were calculated by annexin V^+/PI^- (early apoptotic) and annexin V^+/PI^+ (late apoptotic) cells. Flow cyotometer analysis was performed by using FACS Calliber (Beckman Coulter,Fullerton, CA). To determine the PDE V expression in U266, approximately 50 μg protein was suspended in Laemmli sample buffer (0.1 M Tris-HCl buffer, pH 6.8, 1% SDS, 0.05% -mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue), boiled for 2 minutes, and electrophoresed on SDS-polyacrylamide gels. Gels were electroblotted onto nitrocellulose membranes in a Tris-glycine buffer system. Incubation with indicated antibodies was performed for 2

hours in TBS-Tween 20 (TBST) containing 1% BSA. Blots were washed with TBST and incubated in anti–rabbit horseradish peroxidase (HRP) conjugates for 2 hours in TBST containing 1% BSA.

Results and discussion

In this study, we demonstrated that PDE V selective inhibitor Vardenafil significantly enhances pharmacologic effect of EGCG. Isobologram analysis of EGCG and Vardenafil suggested that the combined treatment with subeffective doses of EGCG and Vardenafil resulted in a synergistic anti-proliferative effect on MM cells. Futhermore, Western blot analysis revealed that U266 cells expressed PDE V .

It was reported that EGCG concentrations used to kill multiple myeloma cells had no effect on survival of normal fibroblasts and peripheral blood mononuclear cells from healthy donors. The authors further suggested that specific killing of multiple myeloma cells was linked to a high expression of the 67-kDa laminin receptor (67LR) (Shammas, *et al,* 2006), which has been shown to be a cell surface receptor for EGCG binding (Tachibana, *et al,* 2004). The 67LR is a non-integrin laminin receptor and known to be overexpressed on the cell surface of various tumour cells (Cioce, *et al*,1991). The expression level of this protein strongly correlates with the risk of tumour invasionand metastasis (Menard, *et al*, 1997). Interestingly, the combination effect of EGCG and Vardenafil was perfectly neutrized by anti-67LR antibody. These results indicated that anti-cancer effect of EGCG and Vardenafil in combination depend on 67LR pathway. The combination significantly induced apoptosis in MM cells but didn't cause any effect on PBMCs which has little 67LR.

Table 1 Anti-cancer effects of EGCG and PDE V selective inhibitor Vardenafil in combination

	Control	Vardenafil	EGCG	EGCG +Vardenafil
Apoptotic Cells (%)	11 ± 1.8	8.7 ± 0.38	11 ± 2.1	42 ± 2.1

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