Suppression of cell proliferation and gene expression by combinatorial synergy of EGCG, resveratrol and γ-tocotrienol in estrogen receptor-positive MCF-7 breast cancer cells

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Abstract. Numerous dietary phytochemicals have shown anti-breast carcinogenic activities when tested *in vitro*; however, in most cases, the demonstrated efficacy of individual phytochemicals requires doses not readily achievable *in vivo*. Therefore, whether diets might exert translational promises and benefits in clinical settings and prevention of breast cancer remain unclear. Since cancer cells are endowed with complex, redundant, converging and diverging pathways spanning both the genetic and metabolic networks that are not merely replicates of those in normal cells, it is of interest to test whether a multicomponent approach involving lower, physiologically relevant doses of natural dietary agents may be developed as a chemopreventive strategy for breast cancer. Herein, we investigated, using the estrogen receptor-positive MCF-7 breast cancer cells as a model, whether the combination of epigallocatechin gallate (EGCG), resveratrol and γ-tocotrienol at suboptimal doses elicits synergism in suppressing cell proliferation, modulating gene expression, and increasing antioxidant activity, as compared to each of the three phytochemicals added alone. The results showed that there was a ~33, 50 and 58% inhibition of cell proliferation by ≥50 μM EGCG, ≥25 μM resveratrol and ≥10 μM γ-tocotrienol, respectively, added as a single agent. When a suboptimal dose (10 μM) of each phytochemical was used, a significant additive effect in suppression of cell proliferation was observed with the combination of resveratrol and γ-tocotrienol whereas the three phytochemicals added together did not produce more pronounced inhibition of cell proliferation. A significant additive effect in reducing cyclin D1 and bcl-2 expression was found when γ-tocotrienol was added with either EGCG or resveratrol. Functional synergism among the three phytochemicals was only observed in the induction of quinone reductase NQO1. These results suggest that diet-based protection against breast cancer may partly derive from synergy amongst dietary phytochemicals directed against specific molecular targets in responsive breast cancer cells, and provide support for the feasibility of the development of a diet-based combinatorial approach in the prevention and treatment of breast cancer.

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Introduction

Breast cancer (BCa) is the most common cancer among women and remains a major health challenge to women in the United States, accounting for >35,000 annual deaths. Most new BCa cases are diagnosed when the tumor is localized; as a result, the majority of these cases (86-96%) have an expected survival of 5 years, with 70 and 53% estimated to have survival extended to 10 and 20 years, respectively. Despite intensive efforts to cure BCa, success in overall treatment has been less than satisfactory, as evidenced by the virtually unchanged age-adjusted mortality from BCa for over 50 years. Moreover, early diagnosis and improved initial treatment options also imply that increasingly, women in the population at large are BCa survivors, at risk for relapse of the disease in which treatment options are notoriously lacking. Thus, prevention and the development of effective therapies are both considered of paramount importance for improving the long-term survival prospect of BCa patients.

The incidence rates of BCa are lowest among Asian women. When Asian women moved to developed host countries and adopted their lifestyle and dietary habits, the risk for BCa is significantly increased. These findings suggest that the Asian diets contain anti-BCa ingredients, possibly, decreased levels of animal fat consumption along with high levels of soy, seaweed, rice, mushrooms, fish and green tea. Case-control, cohort and ecological studies have consistently associated consumption of fruits and vegetables with increased survival of recurrent BCa cases; beneficial effects are evident in the reported 20-90% reduced death risks (1,2), thereby implicating a diet-based strategy as offering promise for treatment and management of BCa. Contrary to translational clinical potential revealed by dietary epidemiological studies, *in vitro* experiments aimed at attempting to replicate anti-BCa activities of Asian diets using single dietary chemicals have produced equivocal results (3-7). It has been proposed that nutritional epidemiology is intrinsically incapable of

identifying all dietary factors as well as their intricate, coordinated interactions with non-nutritional factors relevant to the observed cancer preventative beneficial outcome (8). We surmise that diet-based protection and prevention against epithelial carcinomas may be related to molecular synergy and novel targets among dietary phytochemicals, resulting in a broadened chemopreventive index marked by increased distinct anti-cancer properties, induction of novel targets and synergistic efficacy and a decreased spectrum of untoward effects that optimally target multiple genetic and epigenetic derangements in BCa (9,10). As proof of principle, we have investigated the anti-BCa efficacy of a phytochemical combination involving two well-studied dietary polyphenols, respectively, epigallocatechin gallate (EGCG) from green tea and resveratrol from grape skin, with a fat-soluble vitamin E, γ-tocotrienol. These phytochemicals have been identified as being present in a human diet (11-17), with overlapping and distinct molecular actions and targets. For example, among the phytochemicals proposed for testing, EGCG is the only one that targets epigenetic control by inhibiting DNA methylation (11). Tocotrienols have been shown to interact specifically with enzymes, structural proteins, lipids and transcription factors (17), while resveratrol reportedly inhibits the progression of BCa in mice (18) and elicits a multitude of effects *in vitro* including the suppression of cell proliferation, induction of cell cycle arrest and apoptosis and modulation of gene expression (19-23). Specifically, we have used the estrogen receptor-positive MCF-7 breast cancer cells as a model to test whether the combination of EGCG, resveratrol and γ-tocotrienol at suboptimal doses elicits synergism in suppressing cell proliferation and clonogenicity, modulating gene expression and increasing antioxidant activity, as compared to each of the three phytochemicals added alone.

Materials and methods

EGCG and resveratrol were purchased from LKT laboratories (St. Paul, MN). γ-Tocotrienol was purchased from Cayman Chemical Co. (Ann Arbor, MI). Primary antibodies such as anti-Rb, anti-E2F, anti-cyclin D1, anti-cdk4, anti-bcl-2, anti-bax, anti-actin and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fetal calf serum, MEM (Minimum Essential Medium Eagle), penicillin and streptomycin were purchased from Cellgro, Inc. (Herndon, VA).

Cell culture, growth inhibition assay, colony formation assay and cell morphology. Human MCF-7 cells were obtained from American Tissue Culture Collection (Manassas, VA) and maintained in MEM supplemented with penicillin, streptomycin and 10% heat inactivated fetal calf serum in a humidified atmosphere of 5% CO_2 in air at 37˚C, as previously described (24-26). For cell proliferation assay, MCF-7 cells were plated in 6-well plates at a seeding density of $5x10^4$ cells/ml. EGCG, resveratrol and γ-tocotrienol dissolved in DMSO, were added to the culture media to the final concentration specified in the text. After 72 h, control and treated cells were harvested by trypsinization and cell numbers were determined using a hemocytometer. Cell viability was assayed by trypan blue dye exclusion (24-26). Harvested cells were washed twice with PBS and pellets were stored at -80˚C for further analysis. Colony formation assay was performed as described previously with some modification (25). Cells (400-800 cells/ml, 2ml/well) were added to six-well tissue culture plates containing varying concentrations of EGCG, resveratrol and γ-tocotrienol, alone and in combination, followed by an additional 8 day incubation to allow colonies to form. Colonies were fixed and stained with 1.25% crystal violet, washed extensively to remove excessive dye and imaged by an HP scanner. Quantitative changes in clonogenicity were determined by extraction colonies with 0.5 ml 10% acetic acid and measuring the absorbance of the extracted dye at 450 nm. The experiments were performed in triplicate. Changes on cell morphology by EGCG, resveratrol and γ-tocotrienol alone or in combination were analyzed by microscopy. After 72 h of treatment, cells were viewed and captured using an inverted microscope (Zeiss, Thornwood, NY) under phase-contrast illumination using a 10X objective.

Preparation of cell extracts for immunoblot analysis and enzyme activity assay. For immunoblotting experiments, cells were collected by centrifugation and were lysed in ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1 % SDS, 1 mM dithiothreitol and 10 μl/ml protease inhibitor cocktail). The extracts were centrifuged and the clear supernatants were stored in aliquots at -80˚C for further analysis. To measure the activities of enzymes, control and treated cells were collected by centrifugation and resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA. The cells were sonicated, followed by centrifugation at 13,000 x g for 10 min at 4˚C. The resulting supernatants were collected for the immediate measurement of various enzyme activities. Protein content of cell lysates were determined by Coomassie protein assay kit (Pierce, Rockford, IL) with BSA as standard.

Immunoblotting. The aliquots of lysates (20 μg of protein) were boiled with a sample buffer for 5 min and resolved by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and blocked in TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween-20) containing 3% nonfat dried milk overnight at 4˚C. The blots were incubated with various primary antibodies, followed by incubation for 1 h with appropriate secondary antibodies conjugated to horseradish peroxidase in TBST. Actin expression was used as a loading control. The intensity of the specific immunoreactive bands were detected by enhanced chemiluminescence (ECL), using the manufacturer's protocol (Kirkegared & Perry Laboratories), quantified by densitometry and normalized against loading control actin, as previously described (27).

Assay of superoxide dismutase (SOD), catalase and quinine reductase 1 (NQO1). SOD activity was assayed using a commercially available kit (Fluka, Switzerland). Catalase was assayed according to (28). Briefly, 0.5 ml of potassium phosphate buffer (pH 7.0) and 50 μl of cell lysates were added to a quartz cuvette. The reaction was started by adding 20 μl of H_2O_2 and the decomposition of H_2O_2 was monitored at 25˚C

for 1 min. The activity of catalase was calculated using extinction coefficient (0.0436 $mM^{-1}cm^{-1}$) and expressed as μmol of H_2O_2 decomposed/min/mg protein. Activity of NQO1 was assayed as described in (29), with slight modifications. Briefly, the reaction mixture contained 33 mM potassium phosphate buffer (pH 7.4), 0.18 mM NADPH, 20 μM menadione, 0.02% bovine serum albumin, 0.01% Tween-20 and cell lysates. The reaction was started by the addition of menadione and the oxidation of NADPH was followed spectrophotometrically at 340 nm for 2 min. Assays were performed at 25˚C with and without 20 μM dicoumarol. The dicoumarol-sensitive part of the activity was taken as a measure of the NQO1 activity, which was expressed as μmol of NADPH oxidized/min/mg protein using the extinction coefficient (6.22 $mM^{-1}cm^{-1}$).

Statistical analysis. The results were expressed as mean ± standard deviation (SD). The significance between the control and treated groups was performed by Student's t-test and P-values <0.001 were taken as significant in all the cell growth and colony formation experiments. For enzyme activity assay, differences between groups were assessed by one-way analysis of variance using the SPSS software package for Windows. Statistical significance for inter-group comparisons at P-values <0.001, <0.01 and <0.05 is provided in the figures.

Results

Effect of EGCG, resveratrol and γ-tocotrienol on cell proliferation, viability and colony formation in MCF-7 breast cancer cells. The effect of EGCG, resveratrol and γ-tocotrienol as a single agent on MCF-7 cell growth inhibition was investigated. MCF-7 cells were incubated with 10, 25 and 50 μM of EGCG, resveratrol and γ-tocotrienol for 72 h. Growth and cell viability of MCF-7 cells were determined by hemocytometer. A significant difference was observed in the response of MCF-7 cells to the addition of EGCG, resveratrol and γ-tocotrienol. In the case of EGCG, no inhibition of cell growth was observed until the highest concentration of 50 μM was reached, which suppressed proliferation by ~20-30% without substantially reducing the overall cell viability (Fig. 1A and B). The growth of MCF-7 cells was decreased by resveratrol in a dose-dependent manner; 50 μM of resveratrol elicited a >70% suppression in proliferation and correspondingly, a 10% reduction in cell viability (middle panel, Fig. 1A and B). The most striking inhibition of MCF-7 cell growth resulted from the addition of γ-tocotrienol, which, at 10 μM, already showed >60% reduction in MCF-7 cell growth, with a commensurate 30% decrease in cell viability (right panel, Fig. 1A and B). On the basis of the observed anti-proliferative activity of individual phytochemicals on MCF-7 cells, we hypothesized that EGCG, resveratrol and γ-tocotrienol might also reduce their survival and colony formation potential. This possibility was investigated using the anchorage-dependent clonogenicity assay, based on staining with crystal violet and spectrophotometric quantification of the dye taken up by control and treated cells. EGCG, resveratrol and γ-tocotrienol exerted differential effects on colony formation in MCF-7 cells. Resveratrol and γ-tocotrienol, as single agents, significantly inhibited the ability of MCF-7 cells to form colonies at doses ≥10 μM, whereas the addition of 10 and 25 μM EGCG significantly stimulated the clonogenicity of MCF-7 cells, when compared with the vehicle treated control cells (Fig. 1C). Taken together, results obtained from the aforedescribed assays further confirmed that treatment with γ-tocotrienol had the most pronounced anti-proliferative affect. In contrast, EGCG was minimally effective, while resveratrol showed an intermediate level of growth suppressive activity. These results suggest that each of these dietary phytochemicals may act by a different mechanism, thus raising the possibility that combination of these phytochemicals might result in functional synergy and complementation. Alternatively, however, it is conceivable that cellular antagonism may also be produced when these phytochemicals are presented as a group to cultured MCF-7 cells.

Effect of EGCG, resveratrol, or γ-tocotrienol alone or in combination on cell proliferation, viability and colony formation in MCF-7 breast cancer cells. To test functional synergy or antagonism among EGCG, resveratrol and γ-tocotrienol, the experiments were repeated using suboptimal dose of each phytochemical, alone and in combination. Since 2 out of 3 phytochemicals tested requires minimally 10 μM to show suppression of cell proliferation, a fixed concentration of 10 μM was chosen for the combinational study. Changes in cell growth, viability, colony formation and cell morphology were again used as assays for functional efficacy. Results in Fig. 2A show synergism with the combined addition of EGCG and resveratrol and to a smaller degree, also between resveratrol and γ-tocotrienol, as measured by inhibition of cell proliferation. Interestingly, when cell viability was used to assay synergism, antagonism was observed. For example, while EGCG or resveratrol had no effect on cell viability when added alone or in combination, cell viability was markedly suppressed by the single addition of γ-tocotrienol. Moreover, the addition of EGCG significantly and almost completely reversed the inhibitory effect of γ-tocotrienol on cell viability, while resveratrol only exerted partial effectiveness in reversing the suppression of cell viability by γ-tocotrienol. As expected, the addition of three phytochemicals did not suppress viability of MCF-7 cells (Fig. 2B).

We also used colony formation assays to test functional synergism or antagonism among the three phytochemicals. Results in Fig. 2C show that, while no inhibition occurred in cells treated with 10 μM EGCG alone, the addition of resveratrol or γ-tocotrienol suppressed colony formation by 37 and 50%, respectively. Two-two combined treatment of phytochemicals showed ~70% reduction with resveratrol and γ-tocotrienol, which was not further increased using all three phytochemicals. These results further validate that 10 μM of each phytochemical, alone and in combination, displays anti-proliferative activities (Fig. 2A).

To further ascertain whether functional synergy or antagonism might exist in group addition of EGCG, resveratrol and γ-tocotrienol, morphological changes were analyzed in the control and treated cells. Cells were viewed in an inverted microscope under phase-contrast illumination at x10 magnification (Fig. 2D). Control cells displayed an oblong, polygonal appearance with solid attachment to the substratum

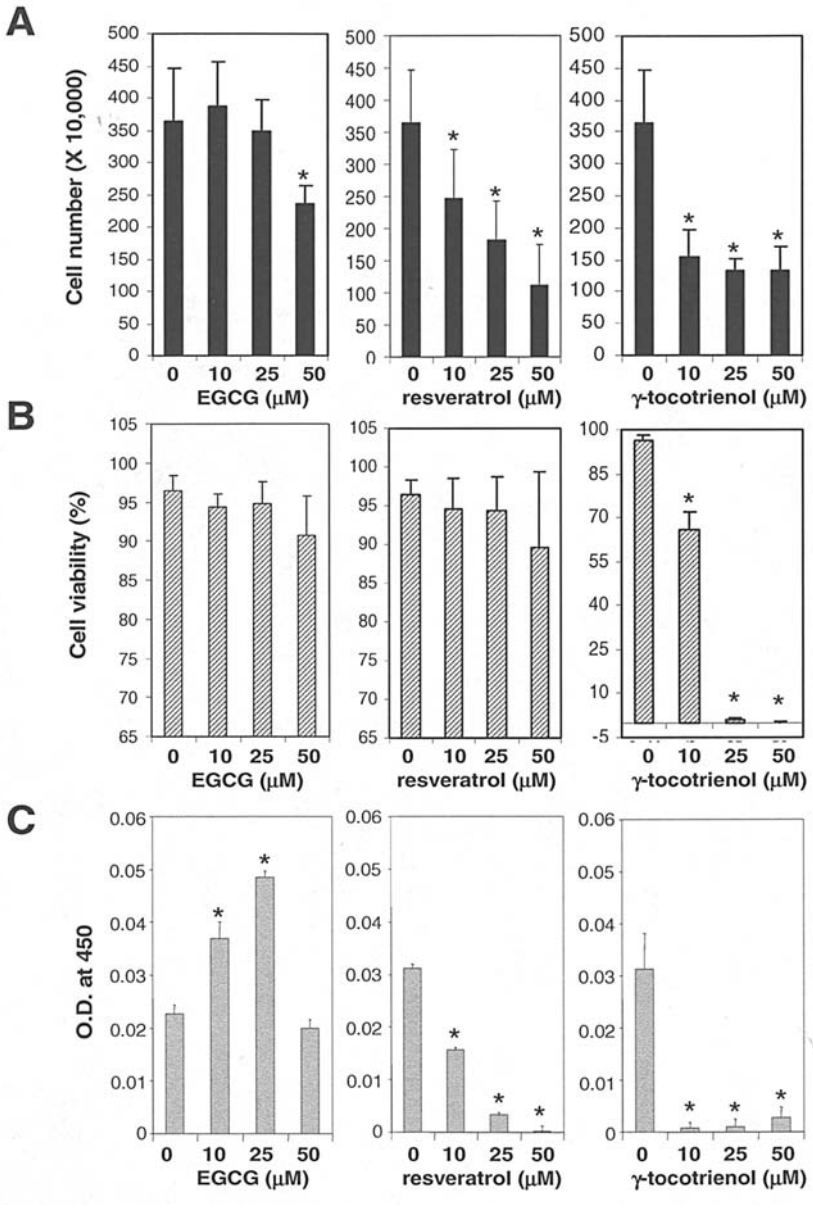

Figure 1. Control of cell growth, viability and colony formation in MCF-7 cells by EGCG, resveratrol and γ-tocotrienol. (A) MCF-7 cells were treated with varying concentrations of phytochemicals (0, 10, 25 and 50 μM). Cell numbers were determined at 72 h by hemocytometer and dose-dependent growth suppression was observed in cells treated with all three phytochemicals. (B) Cell viability was measured using the trypan blue dye exclusion assay. Dose-dependent decrease on cell viability was only observed in γ-tocotrienol-treated cells. (C) Colony formation assay was performed and quantified by spectrophotometer at 450 nm. A dose-dependent decrease on the formation of colony was found in cells treated with resveratrol and γ-tocotrienol. Values are expressed as mean ± SD from three experiments and the symbol represents statistical significance: *P<0.001.

giving them a flattened cobblestone appearance with abundant cytoplasm and only occasional presence of round dead, floating cells in the entire cell populace. Cells treated with EGCG or resveratrol both lowered cell density slightly; EGCG treatment also reduced cell size while cells became noticeably larger in resveratrol-treated cells. When cells were treated with EGCG and resveratrol, more dramatic morphological changes were observed, best illustrated by a greater percentage of cells showing residual shrinkage. The most striking morphological changes and reduction in cell number occurred in cells treated with γ-tocotrienol. Notably, the observed morphological changes in γ-tocotrienol-treated cells became even more pronounced with the addition of EGCG or resveratrol. The addition of all three phytochemicals elicited the most profound cell morphological change. Taken as a whole, these observations further support the notion that functional synergy and complementation may result from exposure of cultured MCF-7 cells to a combination of EGCG, resveratrol and γ-tocotrienol.

Effect of EGCG, resveratrol, or γ-tocotrienol alone or in combination on specific gene expression in MCF-7 breast cancer cells. As single agents, high concentrations (≥50 μM) of EGCG, resveratrol or γ-tocotrienol have been reported to induce apoptosis and cell cycle arrest at G_1 (30-32). Whether sub-optimal doses of these three phytochemicals, alone or in combination, might exert functional synergy in cell cycle control have not been investigated. Since progression through

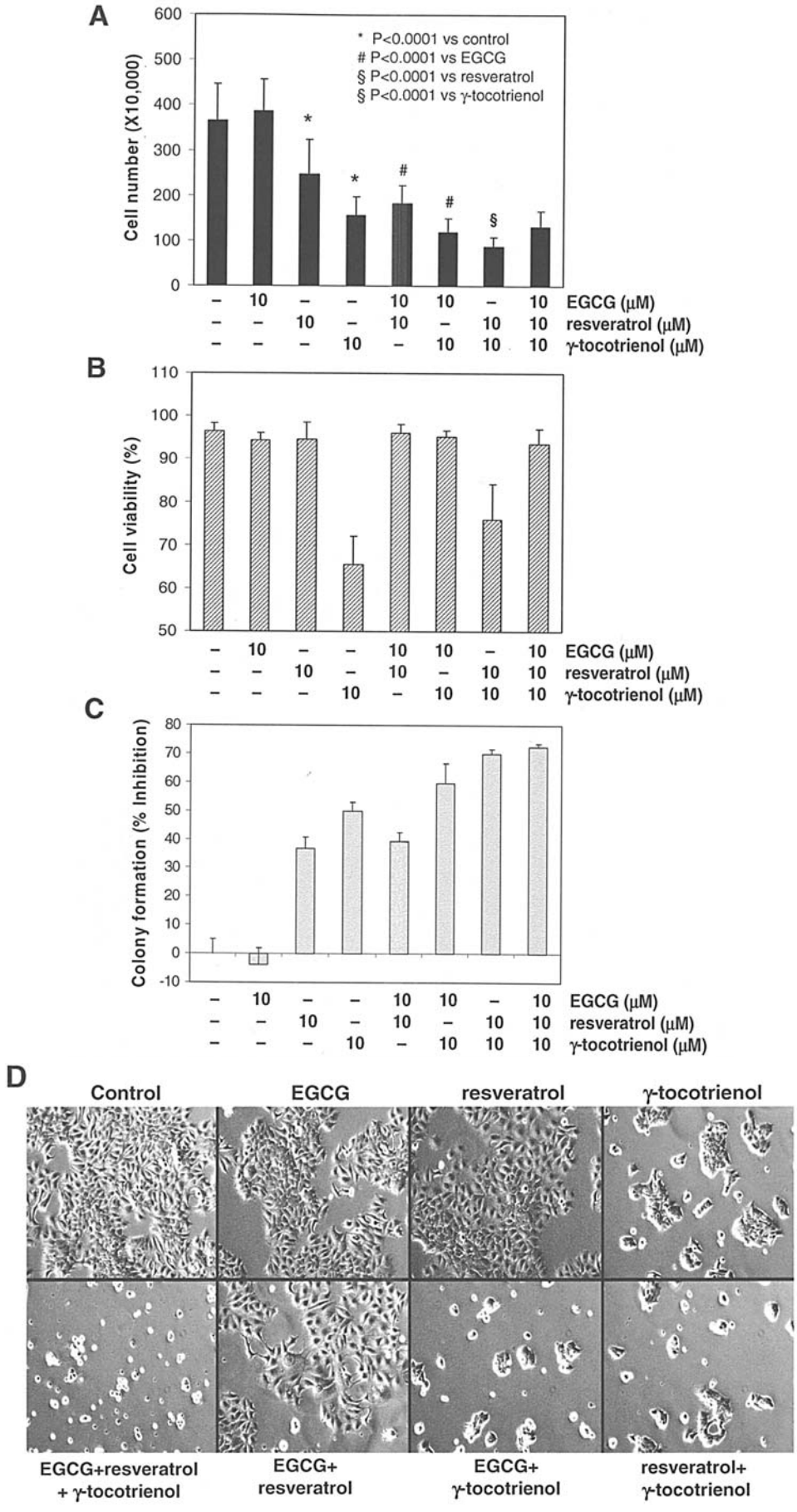

Figure 2. Control of cell growth, viability, colony formation and cell morphology in MCF-7 cells by 10 μM EGCG, resveratrol and γ-tocotrienol alone or in combination of two or three agents. (A) Growth response of MCF-7 cells by different agents. (B) Effects of treatments on cell viability. (C) Effects of treatment on colony formation were quantified and presented as a percentage of control (set as 100%). (D) Changes on cell morphology by different treatments were captured by inverted microscope. Values are expressed as mean ± SD from three experiments and the symbol represents statistical significance.

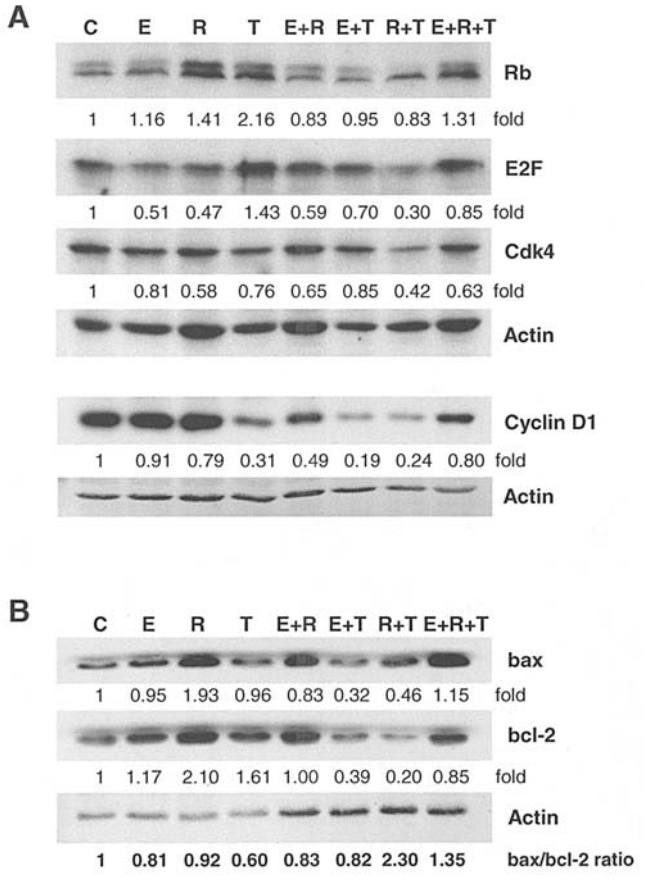

Figure 3. Effects of EGCG (E), resveratrol (R) and γ-tocotrienol (T) alone and in combination on the changes of the various cell cycle regulatory protein expression levels in MCF-7 cells. (A) MCF-7 cells were treated with 10 μM of different combinations of EGCG, resveratrol and γ-tocotrienol for 72 h and the effects on Rb, E2F, cdk4 and cyclin D1 protein expression were determined by Western blot analysis. (B) The total protein expression level of bax and bcl-2 in control and treated MCF-7 were also determined by Western blot analysis. Treatments caused a change in the ratio of bax to bcl-2 has also been calculated. Actin was used as a loading control to determined changes in the expression by treatment. The intensity of the specific immunoreactive bands were quantified by densitometry and expressed as a fold difference against actin.

G_1 checkpoint is primarily controlled by the retinoblastoma Rb/E2F protein complex, we studied changes in Rb/E2F expression by Western blot analysis in control and cells treated for 72 h with 10 μM EGCG, resveratrol or γ-tocotrienol, alone or in combination. Cells treated alone with 10 μM resveratrol or γ-tocotrienol showed an upregulation of Rb and E2F, respectively while EGCG at the same concentration had no effect (Fig. 3A). However, synergistic inhibition on the expression of total Rb but not E2F was observed in cells treated with EGCG combined with either resveratrol or γ-tocotrienol (Fig. 3A). Moreover, an even more significant inhibition on Rb/E2F expression was found in the resveratrol/γ-tocotrienol combination (Fig. 3A). It is noteworthy that when all three agents were co-administered to MCF-7 cells, the inhibition on Rb/E2F expression was less pronounced compared to the two-two phytochemical combination (Fig. 3A). Since cyclin D1 and cdk4 play a pivotal role in controlling Rb/E2F complex, we also measured the expression levels of cyclin D1 and cdk4. Results in Fig. 3A show that cells treated with 10 μM γ-tocotrienol alone significantly reduced cyclin D1 expression, concomitant with a lesser effect on cdk4 level. In EGCG/resveratrol and EGCG/γ-tocotrienol combination-treated cells, inhibitory synergism on the expression of cyclin D1 was observed, while a more pronounced decrease in the levels of cyclin D1 and cdk4 expression was found in cells treated with resveratrol and γ-tocotrienol (Fig. 3A). Indeed, the combined addition of 10 μM resveratrol and γ-tocotrienol showed the most potent effect, compared with other two-two phytochemical combinations tested.

Since these three phytochemicals used alone also induce apoptosis, whether suboptimal dose range of these phytochemicals exerts a synergistic effect in inducing apoptosis was of interest and hence investigated. Both bcl-2 and bax play important roles in apoptosis, respectively, as suppressor and potentiator. Indeed, the ratio of bax to bcl-2 serves to predict the apoptotic potential of various chemotherapeutic and chemopreventive agents. Western blot analysis was performed to investigate changes in the expression of bax/bcl-2 in control and cells treated with each of the three phytochemicals, alone or in combination. Cells treated with 10 μM resveratrol alone caused upregulation of both bax and bcl-2 expression without commensurately changing the ratio of bax to bcl-2 (Fig. 3B). By contrast, upregulation of bcl-2 by 10 μM γ-tocotrienol treatment was accompanied by a decrease of bax/bcl-2 ratio (Fig. 3B). Similarly, possible functional synergism between resveratrol and γ-tocotrienol was inferred by a marked increase in bax/bcl-2 ratio (Fig. 3B). Western blot analysis also showed suppression of bcl-2 expression, simultaneously accompanied by increases in bax, in cells treated with all three agents together (Fig. 3B), as evident by a noted, though smaller increase in bax/bcl-2 ratio (Fig. 3B). These results lend credence to the existence of synergism between EGCG, resveratrol and γ-tocotrienol in the context of activation of the apoptotic cascade.

Effect of EGCG, resveratrol, or γ-tocotrienol alone or in combination on changes in antioxidant enzymes (SOD and catalase) and NQO1 in MCF-7 breast cancer cells. EGCG, resveratrol, or γ-tocotrienol has been studied individually in regard to its antioxidant potential. However, whether the antioxidant activity and capacity of cells might be affected by their combinations at sub-optimal doses remain unclear. Accordingly, we assayed for changes in superoxide dismutase (SOD) and catalase activity as functional read-outs of their potential synergy or antagonism in modulating the antioxidant defense mechanisms. The addition of EGCG or resveratrol had no effect on SOD, while treatment with 10 μM γ-tocotrienol increased SOD by 30%, compared to control cells. Unexpectedly, treatment with the phytochemical combination in groups of two or three attenuated the increase in SOD (Fig. 4A). With respect to changes in catalase, no effect was found in cells treated with single phytochemical alone (Fig. 4B). The catalase activity was suppressed in cells treated with EGCG and resveratrol and increased with the EGCG/ γ-tocotrienol combination (Fig. 4B). The addition of all three phytochemicals to cultured MCF-7 cells failed to alter the catalase activity (Fig. 4).

NQO1 activity was also measured in cells treated with single or combined phytochemicals. Both EGCG and

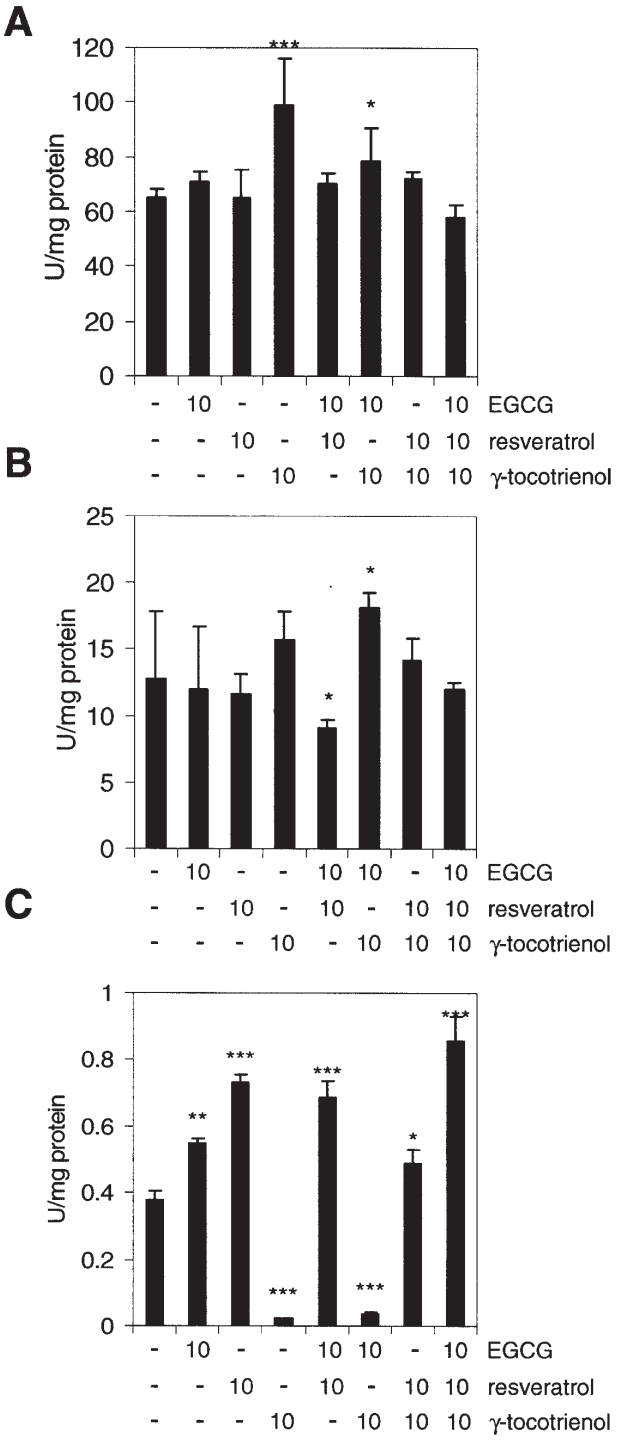

Figure 4. Activities of antioxidant enzymes and NQO1. MCF-7 cells were treated with different combinations of EGCG, resveratrol and γ-tocotrienol (10 μM) for 72 h and the cells were analyzed for (A) SOD, (B) catalase and (C) NQO1 activity. Values are expressed as mean ± SD for three experiments. Units: SOD, amount of enzyme that inhibits the activity of xanthine oxidase by 50%; Catalase, μmol of H_2O_2 decomposed/min; NQO1, μmol of NADPH oxidized/min/mg protein Comparisons are made between control and different treatments. The symbols represent statistical significance: *P<0.05, **P<0.01 and ***P<0.001.

resveratrol markedly increased NQO1, whereas the NQO1 activity was significantly decreased by γ-tocotrienol (Fig. 4C). The combination of EGCG and resveratrol did not elicit further increases in NQO1 (Fig. 4C). With cells treated with both resveratrol and γ-tocotrienol, γ-tocotrienol was found to completely reverse the induction of NQO1 by resveratrol (Fig. 4C). By comparison, γ-tocotrienol did not have any effect on the induction of NQO1 by EGCG (Fig. 4C). A functional synergism was observed on the induction of NQO1 by the addition of three compounds together (Fig. 4C). These results provide support for the notion that when MCF-7 cells are exposed to a combination of EGCG, resveratrol and γ-tocotrienol, functional synergy and complementation might ensue.

Discussion

Epidemiological studies have shown that incidence rates of BCa are lowest among Asians, which suggest that Asian diets contain anti-BCa ingredients. *In vitro* studies demonstrated that phytochemicals derived from diets show activity for the prevention and treatment of human BCa; however, *in vitro* studies involving phytochemicals alone mostly have failed to convincingly show that the efficacious doses are not achievable physiologically therefore casting into doubt whether the observed *in vitro* activities are within the realm of what are considered translationally relevant or beneficial in clinical settings.

We postulate that diet-based protection against BCa may derive from molecular synergy that expands the framework of distinct anti-carcinogenic activities and at the same time reduces the untoward adverse effects. A relatively low 10 μM dose was chosen for the present studies. Such a dose, while not likely to be physiologically achievable as pharmacokinetic studies have reported that the human plasma concentration of EGCG and resveratrol appeared only transiently at ≤1 μM and ~2.4 μM, respectively (33-36), are considerably lower than the efficacious dose of 20 to 100 μM used for most *in vitro* studies (34,35,37) and indeed, might be close to physiological relevance if one assumes that phytochemicals operate mechanistically at the responsive cells by the 'hit-and-commit' principal.

Using MCF-7 cells as a model, we demonstrated functional synergism between suboptimal 10 μM resveratrol and γ-tocotrienol, as assayed by suppression of cell proliferation, downregulation of cell cycle regulatory proteins, notably, E2F, cdk4 and cyclin D1, as well as upregulation of apoptosis gene expression, evident in the measured ratio of bax-to-bcl-2. It is interesting that when all three phytochemicals are co-administered together, antagonism rather than synergism was frequently observed. These results imply that whether the combinatorial cocktail approach might show promise for prevention and treatment, it is still a challenging task to *a priori* predict whether a composite combination would lead to synergistic or antagonistic interaction. Therefore, further investigations to identify the molecular targets and elucidate the mechanisms underpinning the observed synergism or antagonism between chemopreventive phytochemicals are imperative and actively pursued in our laboratory.

Our studies also show that the three phytochemicals act as potent inducers of quinone reductase NQO1. NQO1 is a main phase II detoxification enzyme that catalyzes the two-electron reductions of a variety of quinone compounds, thereby

conferring cellular protection against potentially mutagenic and procarcinogenic free radicals and toxic oxygen metabolites generated by the cytochrome P450 effected one-election reductions (38). In addition to its well studied role in detoxification and protection against carcinogenic xenobiotics, NQO1 also serves as an ancillary role in chemotherapy as it is capable of converting certain quinones into cytotoxic anti-tumor quinones via bioreduction (39-42). For example, induction of NQO1 by dietary means has been shown to enhance the chemotherapeutic efficacy of mitomycin C and ß-lapachone (39-42). In this study, we found that the combination of EGCG, resveratrol and γ-tocotrienol together at suboptimal dose (10 μM) resulted in the synergistic induction of NQO1. These results not only reinforce the translational application of this cocktail in chemoprevention of BCa but also imply that the cocktail may have an expanded therapeutic scope for BCa treatment and management, via the NQO1-dependent activation of prodrugs.

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