LIPID RAFTS MEDIATE EPIGALLOCATECHIN-3-GALLATE- AND GREEN TEA EXTRACT-DEPENDENT VIABILITY OF HUMAN COLON ADENOCARCINOMA COLO 205 CELLS; CLUSTERIN AFFECTS LIPID RAFTS-ASSOCIATED SIGNALING PATHWAYS

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Epigallocatechin-3-gallate (EGCG) is an important bioactive constituent of green tea extract (GTE) that was widely believed to reduce proliferation of many cancer cell lines. The purpose of this study was to verify the possible pro-apoptotic action of GTE/EGCG in human colon adenocarcinoma COLO 205 cells. The effect of EGCG/GTE treatments on cell viability was studied using methyl thiazolyl tetrazolium (MTT) assay. Cell proliferation was assessed with crystal violet staining, whereas protein expression levels were evaluated by western blotting followed by densitometric analysis. Obtained results were analyzed statistically. Surprisingly, EGCG/GTE dose-dependently up-regulated COLO 205 cells viability and proliferation. Observed effects were mediated by lipid rafts, as cholesterol depletion significantly prevented EGCG/GTE-dependent cell survival. Furthermore, treatment of COLO 205 cells with EGCG/GTE resulted in activation of MEK/ERK1/2, but not Akt1/2/GSK-3β signaling pathway. The presence of MEK inhibitor - PD98059 but not PI3-K inhibitor - LY294002, both reduced EGCG/GTE-induced ERK1/2 activation and the proliferative effect of catechins. Furthermore, EGCG/GTE stimulated secretory clusterin (sClu) expression level, which underwent complex control through lipid rafts/PKC/Wnt/β-catenin system. Our studies demonstrated that EGCG and GTE stimulate cell survival and proliferation of COLO 205 cells in a lipid rafts-dependent manner via at least MEK/ERK1/2 signaling pathway. Furthermore, EGCG/GTE mediated positive effects on viability and mitogenicity of COLO 205, while suppression of β-catenin activity was positively correlated with sClu clusterin expression.

Key words: apoptosis, clusterin, COLO 205 cells, epigallocatechin gallate, ERK1/2, green tea extract
and other boronic acid-based drugs in multiple myeloma cells. Bearing in mind that green tea is a world-wide popular beverage, its pro-/anti-cancer properties should be tested in cell-type dependent context. Hitherto, there are no data concerning the molecular mechanism of EGCG and GTE action in human colon adenocarcinoma COLO 205 cells. It should be noted that the COLO 205 cell line represents extremely resistant phenotype and it neither responds to death ligands nor to anti-cancer drugs-induced death signals (16). According to Niedzielska et al. (17) growth factors, oncogenic cytokines, such as IL-2, IL-12, IFN-gamma, TRAIL and TNF-α, play a crucial role in the growth and survival of premalignant colonic tissue and developed tumor, instead of apoptosis induction. However, we found that some compounds are able to sensitize COLO 205 cells to TNF-α-induced apoptosis or initiate cell death itself (18, 19). The aim of the present study was to verify whether EGCG and/or GTE could eliminate COLO 205 cells and which molecular mechanisms determine EGCG/GTE biological effects.

MATERIALS AND METHODS

Reagents

All reagents: dimethyl sulfoxide (DMSO), Tris, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), (2-aminoethoxyethane)-N,N,N',N'-tetraacetic acid (EGTA), polyoxymethylene sorbitan monolaurate (TWEEN 20), sodium chloride (NaCl), bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethylsulphonylfluoride (PMSF), dithiothreitol (DTT), paraformaldehyde, cycloheximide (CHX), actinomycin D (AD), staurosporine (STS), methyl-β-cyclodextrin (MβCD), lithium chloride (LiCl), green tea extract (GTE, Polyphenon E), epigallocatechin gallate (EGCG), (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) (PD98059), N-(2-aminoethoxy)-5-isoquinolinesulfonamide dihydrochloride (H9), 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763), phosphorol 12-mycristate 13-acetate (PMA), D-mannitol, sucrose, crystal violet (CV), ipegal CA-630, nystatin (NY), imipramine (IMP) were cell culture tested, of high purity, and unless otherwise stated purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Reagents for experimental use were prepared according to the manufacturer's recommendations and if possible stored as stock solutions (1000-fold the highest working concentration). All primary antibodies, except mouse anti-clusterin (Upstate, Lake Placid, NY, USA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary horseradish peroxidase (HRP) conjugated donkey anti-rabbit and donkey anti-goat were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary horseradish peroxidase (HRP) conjugated donkey anti-rabbit and donkey anti-goat were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas donkey anti-mouse HRP Ab were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Other reagents were purchased as stated in the description of the respective methods (see the text). Sodium dodecyl sulphate (SDS) 100 g/L, sequi-blot polyvinylidene fluoride (PVDF) membrane 0.2 µm and all reagents for immunoblotting were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Sera, media and antibodies were obtained from Gibco Life Technologies (Paisley, United Kingdom).

Cell culture

Human colon adenocarcinoma cell line COLO 205 was purchased from American Type Culture Collection (ATCC). Cells were maintained in the exponential phase of growth in growth medium [GM, 100 mL/L fetal bovine serum (FBS)/Dulbecco's Modified Eagle Medium (DMEM) with glutamax and antibiotic-antimycotic mixture (penicillin G sodium salt 50 IU/mL, streptomycin sulphate 50 µg/mL, gentamycin sulphate 20 µg/mL, fungizone-amphotericin B 1 µg/mL)]. The cells were grown at 37°C, in a controlled, humidified 50 mL/L CO₂ atmosphere, on 96-well flat-bottomed or tissue culture Petri dishes (100 mm diameter, BD Biosciences Pharmingen, San Jose, CA USA).

Experimental procedure

During propagation, the medium was changed every other day until cultures reached 100% confluence. One-day (24 h) prior to the experiment, confluent cells (cells of the same cell density fully covering the surface of the dish) were then switched to post-mitotic status to induce quiescence (withdrawal from cell cycle) by replacing GM with 20 g/L BSA/DMEM designated as a control medium (CTRL). In the above-mentioned conditions divisions of COLO 205 cell have been completed. Foundations for the collection time-points (6, 12, 24, 48 hours) and concentration range were established in preliminary experiments. During the study freshly prepared media with or without experimental factors had been changed according to the experimental schedule.

Cell viability

Cell viability based on mitochondrial function was assayed by the ability of cells to convert soluble MTT (3-(3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into an insoluble purple formazan reaction product with minor modifications to protocol described by Jacobson (20). For this assay, during the last hour of incubation the media were replaced with MTT solution (5 mg/mL in DMEM without phenol red, sterilized by filtration). MTT solution was then aspirated and formazan in cells was instantly dissolved by the addition of 100 µL DMSO. Before the application of MTT, cells were examined under phase-contrast microscopy to visually assess the degree of cell death. The absorbance was measured at 570 nm with ELISA reader type Infinite 200 (TECAN, Austria). Percentage of viable cells was measured by MTT conversion into purple formazan either in 20 g/L BSA/DMEM or 1 mL/L DMSO and was related to mitochondrial respiration or activity of mitochondrial dehydrogenases.

Cell proliferation

Cells were grown on 96-well plates until they reached about 50% confluence. One-day (24 h) prior to the experiment cells were switched to post-mitotic status to induce quiescence by replacing GM with 20 g/L BSA/DMEM designated as a control medium (CTRL). Further, cells were treated for 24 hours with tested compounds as this period was previously shown to evoke marked changes in mitogenicity. After the experiment, cell proliferation was recovered by adding fresh GM and cells were incubated for 24 hours. Next, cells were fixed twice with 75% and 100% ice-cold methanol for 20 min. The plates were then stained with 50 µL of 0.2% CV in 2% EtOH/H₂O v/v solution. After 10 min incubation, excess dye was removed by washing with distilled water and the plates were air dried before extraction of the bound dye with 1% SDS solution. Optical density of the dye extracts was measured at 570 nm with ELISA reader type Infinite 200 (TECAN, Austria).

Postembedding immunostaining

For immunocytochemical studies the cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 h at 4°C. Next, cells were washed with the same buffer
and post-fixed with 1% OsO₄ for 1 hour. After dehydration cell were embedded in Epon 812 and ultrathin sections were processed according to the post-embedding procedure. The sections were mounted on the formvar-coated nickel grids, placed in 10% hydrogen peroxide for 10 min, rinsed in PBS for 30 min and further incubated with 5% BSA in PBS for 10 min. For single labeling mouse monoclonal anti-clusterin IgG (Upstate, Lake Placid, NY, USA) was diluted 1:20 in PBS. After 24 h at 4°C the grids were washed in PBS for 30 min and exposed to secondary mouse anti-mouse IgG conjugated with colloidal gold particles of 18 nm in diameter (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:20 in PBS. After 1 h incubation in darkness at RT the grids were washed with PBS for 15 min, followed by distilled water for 15 min. Ultrathin sections were air-dried, and stained with 4.7% uranyl acetate for 10 min and with lead citrate for 2 min. The sections were examined and photographed with a JEOL JEM 1011 electron microscope (Jeol, Tokyo, Japan).

Isolation of cytoplasmic and nuclear fractions

Cells were grown on 100 mm diameter culture Petri dishes. Following each experiment cells were washed twice with PBS, scraped off in PBS and spun down (10000 g for 5 min, 4°C). Cell pellets were stored at -80°C until the end of the experiment. Cell pellets were resuspended in 400 µL of ice-cold buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), then were incubated on ice for 15 min, after which 25 µL of a 100 mM/L solution of Igepal CA-630 was added. After centrifugation (RT, 10000 g, 30 s), supernatants containing cytoplasm were transferred to fresh tubes and were stored at -20°C. Nuclear pellets were resuspended in 200 µL RIPA buffer (1x PBS, 10 mL/L Igepal CA-630, 5 g/L sodium deoxycholate, 1 g/L SDS, 0.4 mM PMSF, 10 µg/mL of aprotinin and 10 µg/mL of sodium orthovanadate) and were passed through a 21-gauge needle. After centrifugation (10,000 g for 5 min, 4°C) nuclear lysates were stored at -80°C until analysis. Soluble protein concentration in the lysates was determined by a protein-dye-binding method (21) with a commercial reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Sample preparation for electrophoresis and immunoblotting

Rafts were isolated by extraction with Brij 98 at 37°C, as previously reported by Drevot et al. (22) with modification of Remacle-Bonnet et al. (22). In brief, cells were harvested with a rubber policeman and gently sonicated (five 5-second bursts, 5 W) in 1 mL of ice-cold buffer A (25 mM/L HEPES, 150 mM/L NaCl, 1 mM/L EGTA, 10 mM/L NaF, 5 mM/L L- NaVO₃, 10 mM/L Na-P, and a mixture of protease inhibitors). The postnuclear supernatant was recovered after centrifugation at 800 g at 4°C for 10 minutes, and then spun at 100,000 g in a SW41 rotor (Beckman Instruments) for 60 minutes at 4°C and the supernatant was referred as the soluble fraction (S) containing solubilized membrane and cytosolic fractions. The membrane raft fraction in the pellet (R) was resuspended in buffer A containing 1% Brij 98, 0.3% deoxycholic acid, and 60 mM/L n-octyl-β-D-glucopyranoside (ODG), and nonsoluble material was removed by an additional centrifugation. Collected (S) and (R) fraction from an identical number of cells were analyzed by WB. Finally, for protein quantification, blots were scanned and analyzed by spot densitometry, and results were expressed as average value of pixels enclosed (AVG), calculated as the sum of all the pixel values after background correction divided by area. To obtain whole-cell lysates an aliquot of 1 mL of ice-cold PBS was added and cells were immediately scraped from the plastics and collected by centrifugation (10,000g for 10 min, 4°C). An aliquot of 1.0 mL of RIPA buffer was added to suspend the cells, which were further broken up by repetitive mixing with the syringe with attached needle (21G, 0.8 mm diameter). Cell lysates were left on ice (4°C) for 30 min, and centrifuged for another 5 min (4°C, 10,000g). The resulting viscous solution was divided into smaller volumes and transferred to fresh Eppendorf tubes and stored at -80°C until used. Soluble protein concentration in the lysates was determined by a protein-dye-binding method (20) with a commercial reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Electrophoresis and immunoblotting

Equal amounts of sample protein (either 50 µg or 30 µg) isolated from the treated or untreated COLO 205 cells were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. The electrotransfer of proteins to PVDF membranes (0.2 µm) was performed for 1.5 hour at 100 V and followed by overnight blocking (4°C) in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) supplemented with 50 g/L non-fat powdered milk. After washing in TBS (TBS containing 0.5 mL/L Tween 20), the membranes were immunostained by standard method provided by the manufacturer (Santa Cruz, CA, USA). They were probed with a primary antibody (mouse monoclonal anti-clusterin, rabbit polyclonal anti-α/α, anti-procaspase-3β, anti-P(Ser473)Akt1/2, anti-α-SK-3β, anti-P(Y204)ERK1/2, anti-ERK1/2 and goat polyclonal anti-P(Ser9)GSK-3β, anti-Akt1/2, anti-PCNA, anti-α/α, anti-catenin, anti-actin antisemur) (Santa Cruz, CA, USA) for 1 hour at 20°C or overnight at 4°C, washed three times in TBST and were further incubated with the secondary donkey anti-rabbit or anti-goat or anti-mouse antibody conjugated with HRP (see Reagent's section). Membranes were also probed with goat polyclonal anti-actin (for whole-cell or cytoplasmic lysates) or anti-PCNA (for nuclear lysates) antibody to normalize protein levels. The blots were developed using the enhanced chemiluminescence (ECL) detection system (Amershams International, Aylesbury, U.K.) according to the manufacturer's protocol. After exposure, photographs were taken with a Kodak DC 290 zoom digital camera and were scanned and analyzed using the Kodak EDAS 290/Kodak 1D 3.5 system.

Statistical analysis

Each treatment was carried out in triplicate and each experiment was repeated at least three times with similar results. The results were statistically evaluated with one-way ANOVA and post-hoc Tukey's multiple range test when compared to control treatments. These analyses were performed using GraphPad Prism™ version 4.03 software (GraphPad Software Inc., San Diego, CA, USA). In order to show the quantitative differences, the percentage of initial control value was set arbitrarily as 100% (experimental value/initial control value x 100) at each time point used. Statistical differences were interpreted as significant at P<0.05 and highly significant at P<0.01.

RESULTS

Epigallocatechin-3-gallate and green tea extract stimulate COLO 205 cells survival and proliferation via lipid rafts-dependent and independent mechanisms

Examination of human colon adenocarcinoma COLO 205 cells revealed that EGCG [10-200 µM] (Fig. 1A) similarly to GTE [10-200 µg/mL] dose- and time-dependently stimulated
repeated at least three times with similar results. Data are representative values. The stimulatory effects of EGCG was detected at 24th hour of experiment and amounted to observed, as confirmed by Western blot analysis and (abrogated and were not significantly different from control cells, and GTE on cell viability and mitogenicity were totally determination of procaspase-3 level (Fig. 1E) of EGCG/GTE action cell viability returned to [200 µg/mL] treatment, respectively. After additional 24 hours (48th hour) of EGCG/GTE action cell viability returned to [200 µg/mL] treatment (6, 12, 24 and 48 h) on viability of COLO 205 cells. (b) Lipid rafts modulators inhibit EGCG-dependent stimulatory effect. Fig. 1A. In GTE- and EGCG-treated cells apoptosis was not observed, as confirmed by Western blot analyses of whole-cell lysates obtained from COLO 205 cells. Immunoblots showing the expression of P(Ser473)-Akt1/2, Akt1/2, P(Ser9)-GSK-3β, GSK-3β, P(Y204)-ERK1/2, ERK1/2 and procaspases-3 after EGCG [100 µM] or GTE [100 µg/mL] treatment. Membranes were also probed with goat polyclonal anti-actin antibody to normalize protein level. Each experiment was repeated at least three times with similar results. Data are representative values.

cell survival (Fig. 1A). The highest COLO 205 cells viability was detected at 24th hour of experiment and amounted to 172.28%±8.93 and 136.96%±7.0 for EGCG [200 µM] and GTE [200 µg/mL] treatment, respectively. After additional 24 hours (48th hour) of EGCG/GTE action cell viability returned to control value. Further, crystal violet staining showed that EGCG [100 µM] or GTE [100 µg/mL] treatments significantly increased cell proliferation up to 122.24%±1.25 for EGCG and 117.96%±2.68 for GTE, in comparison to control cells (P>0.05) (Fig. 1C). In GTE- and EGCG-treated cells apoptosis was not observed, as confirmed by Western blot analysis and determination of procaspase-3 level (Fig. 1E). Based on MTT assay, 100 µM EGCG and 100 µg/mL GTE concentrations were chosen for further analyses. The stimulatory effects of EGCG and GTE on cell viability and mitogenicity were totally abrogated and were not significantly different from control cells, when lipid rafts modulators such as methyl-β-cyclodextrin (MβCD, 0.2 mM for 24 h or 2 mM for 1 h preincubation), nystatin (NY, 10 µg/mL, 1 h preincubation) or imipramine (IMP, 10 µM, 1 h preincubation) were present (Fig. 1B, IC). It was previously shown that positive effects of EGCG/GTE are mediated by PI3-K/Akt1/2/GSK-3β and/or MEK/ERK1/2 signaling pathways (10). As shown on Fig. 1E in COLO 205 cells EGCG and GTE did not affect significantly P(Ser473)-Akt1/2 levels calculated as ratio of OD P(Ser473)-Akt-1/2/Akt1/2 levels calculated as ratio of OD P(Ser473)-Akt1/2/Akt1/2 (Fig. 1E). Similarly, P(Ser473)-Akt1/2 expression levels were stable in untreated cells. At 24th hour of treatment either with EGCG or GTE there was rise in P(Ser473)-Akt-1/2/Akt1/2 and drop in P(Ser9)-GSK-3β levels known downstream target of P(Ser473)-Akt1/2. suggesting other than P(Ser473)-Akt1/2-dependent mechanism contributed to GSK-3β (Ser9) phosphorylation in COLO 205 cells (Fig. 1E). EGCG and GTE presence affected also the phosphorylation status of ERK1/2 kinase at Y204 position, which was time-dependent and increased up to 48th hour of experiment (Fig. 1E). OD ratio of P(Y204)-ERK1/2/ERK1/2 amounted to 0.932±0.018 for EGCG and 0.384±0.016 for GTE at 24th hour of experiment and was significantly different from ratio calculated at 6th hour of experiment (0.447±0.008 and 0.259±0.018 for EGCG and GTE, respectively). When cholesterol was depleted from lipid rafts by
influence of PD98059 (50 µM) (Fig. 2A) or GTE [100 µg/mL]+PD98059 [50 µM] (Fig. 2C) in comparison to untreated cells. Membranes were also probed with goat polyclonal anti-actin antibody to normalize protein level. Each experiment was repeated at least three times with similar results. Data are representative values.

Fig. 2. (a) Cholesterol deprivation affects EGCG- and GTE-mediated biological effects. Western-blot analyses of whole-cell lysates obtained from COLO 205 cells. Immunoblots showing the expression of P(Ser473)-Akt1/2, Akt1/2, P(Ser9)-GSK-3β, GSK-3β, P(Y204)-ERK1/2, ERK1/2 and procaspases-3 after MβCD [0.2 mM] or EGCG [100 µM]+MβCD [0.2 mM] or GTE [100 µg/mL]+MβCD [0.2 mM] treatment in comparison to untreated cells. Membranes were also probed with goat polyclonal anti-actin antibody to normalize protein level. Each experiment was repeated at least three times with similar results. Data are representative values. (b) Inhibition of MEK kinases inhibits EGCG or GTE stimulatory effects and decreased COLO 205 cells viability. MTT assay showing influence of PD98059 [50 µM] (●) or EGCG [100 µM]+PD 98059 [50 µM] (□) or GTE [100 µg/mL]+PD98059 [50 µM] (▲) (6, 12, 24, 48 h) treatment on viability of COLO 205 cells. Significant differences between the treatment means and control values at respective times are indicated by * P<0.05, ** P<0.01 and *** P<0.001. Values represent means from three identical experiments carried out in quadruplicates ±S.E.M. (c) Western-blot analyses of whole-cell lysates obtained from COLO 205 cells. Immunoblots showing the expression of P(Ser473)-Akt1/2, Akt1/2, P(Ser9)-GSK-3β, GSK-3β, P(Y204)-ERK1/2, ERK1/2 and procaspases-3 after PD98059 [50 µM] or EGCG [100 µM]+PD98059 [50 µM] or GTE [100 µg/mL]+PD98059 [50 µM] treatment. Membranes were also probed with goat polyclonal anti-actin antibody to normalize protein level. Each experiment was repeated at least three times with similar results. Data are representative values.

MβCD [0.2 mM] the EGCG/GTE-dependent ERK1/2 phosphorylation was differently modulated. As presented on Fig. 2A, MβCD [0.2 mM] added to EGCG/GTE treatment reduced the levels of P(Ser473)-Akt1/2 and P(Y204)-ERK1/2 in comparison to EGCG or GTE alone (Fig. 2A vs. Fig. 1E). To verify the importance of particular signaling pathway in EGCG/GTE positive action on cell viability and mitogeneity, the specific metabolic inhibitors: LY294002, PI3-K inhibitor and PD98059 - MEK1 inhibitor were used. We found that irrespective to LY294002 concentration [up to 100 µM], P(Ser473)-Akt1/2 protein expression level was not affected as we showed previously (24) suggesting relative resistance of COLO 205 cell line to LY294002 inhibitor. Accordingly, PI3-K inhibition did not affect EGCG/GTE action on cell viability. In contrast, the blockade of MEK by PD98059 [50 µM] was followed by reduced ERK1/2 phosphorylation at Y204 position even in the presence of GTE/EGCG (Fig. 2A). Substantial drop in cell viability upon PD98059 administration regardless of GTE/EGCG presence confirms that catechins acted through the MEK/ERK1/2 signaling pathway. Moreover, chronic inhibition of ERK1/2-dependent pathway, up to 48 hours resulted in extensive cell death demonstrated via decline of cell viability (Fig. 2B, 2C). Apart from the activation of cellular pro-survival pathways, EGCG and GTE were described to modulate the balance between pro-/anti-apoptotic Bcl-2 family proteins, particularly Bcl-2 and Bax and to induce antipapoptotic Bcl-2 protein (10). We performed Western blot analysis and evaluated the expression of certain members of Bcl-2 family (Bcl-2, Bax, Bak, XIAP, survivin) after EGCG/GTE treatment (6, 12, 24 and 48 h), however, we did not observe any significant changes in
the proteomic profile analyzed (data not shown). Thus, we assumed that other apoptosis-regulating proteins or non-conventional apoptosis (calpain-mediated apoptosis?) should mediate EGCG/GTE action.

Green tea extract and epigallocatechin-3-gallate induce clusterin expression in COLO 205 cells

Clusterin is among proteins which are indicated as targets for EGCG action. Thus, we performed Western blot analysis to examine the expression levels of clusterin after EGCG/GTE treatments. As shown on Fig. 3A, EGCG [100 µM] and GTE [100 µg/mL] transiently and markedly elevated secretory clusterin (sClu, 40 kDa) expression at 12th hour of experiment. Interestingly, higher sClu expression was no longer detected after subsequent 12 or 24 hours of EGCG/GTE treatment. The sClu expression after EGCG [100 µM, 12 h] administration was confirmed by the postembedding immunocytochemistry. It allowed detection of gold particles representing clusterin in endosomes and at vicinity of plasma membrane (Fig. 3B).

Complex regulation of clusterin expression in COLO 205 cells after epigallocatechin-3-gallate/green tea extract treatment

There are several contradictory data concerning the mechanisms of clusterin expression in cancer cells. To identify the possible pathways responsible for clusterin up-regulation after EGCG/GTE treatments in COLO 205 cells, a wide range of metabolic inhibitors in the presence/absence of EGCG/GTE were used. Western-blot analysis revealed that the additional presence of PKA inhibitor - H9 [10 µM] or MEK inhibitor - PD98059 [50 µM] did not modulate the EGCG [100 µM] or GTE [100 µg/mL] action on sCLU expression (Fig. 4A). Inhibition of PKC isoforms by a long-term treatment with PMA [100 nM] or GSK-3β by SB216763 [10 M] postponed sClu induction until 24th hour of experiment but high sClu levels remained for the additional 24 hours (Fig. 4B). On the other hand, the use of LiCl, known GSK-3β inhibitor [LiCl, 20 mM] strengthened EGCG/GTE action manifested by clusterin up-regulation as early as 6th hour of experiment (Fig. 4C). The most potent effects on sClu expression were revealed with PKC inhibition by staurosporine (STS, 1 µM), cholesterol deprivation with MβCD [0.2 mM] and retarded translation by cycloheximide treatment (CHX, 1 µg/mL. Administration of STS or CHX blocked EGCG/GTE to induce sClu expression (Fig. 4D), whereas MβCD counteracted EGCG but not GTE action. After simultaneous GTE [100 ng/mL] and MβCD [0.2 mM] treatment, sClu expression was up-regulated, however sClu remained at considerably lower level than in solely GTE-treated cells (Fig. 4D). The latter observation might be associated with the changes in plasma fluidity and altered sCLU secretion, but the concentration of MβCD used by us does not significantly affect cell viability (23). Lack of EGCG/GTE-induced sClu expression in the presence STS, CHX or MβCD correlated with retarded cell proliferation, which was not stimulated by EGCG or GTE, anymore (Fig. 5A). According to crystal violet staining, LR modulators significantly reduced EGCG/GTE stimulatory effects (122.24%±1.25 and 119.21%±1.78, respectively) as cell proliferation amounted to 95.90%±3.71 and 92.08%±1.91 for EGCG/GTE+STS [1 µM]
could significantly affect its action (27). To verify our assumption, lived protein, thus the presence of translation inhibitor - CHX µM]+PD98059 [50 µM] or GTE [100 µg/mL]+PD98059 [50 µM]; (b) PMA [100 nM] or EGCG [100 µM]+PMA [100 nM] or GTE (a) H9 [10 µM] or EGCG [100 µM]+H9 [10 µM] or GTE [100 µg/mL]+H9 [10 µM] or PD98059 [50 µM] or EGCG [100 µM]+PD98059 [50 µM]; (b) PMA [100 nM] or EGCG [100 µM]+PMA [100 nM] or GTE [100 µg/mL]+PMA [100 nM] or SB216763 [10 µM] or EGCG [100 µM]+SB216763 [10 µM] or GTE [100 µg/mL]+SB216763 [10 µM]; (c) LiCl [20 mM] or EGCG [100 µM]+LiCl [20 mM] or GTE [100 µg/mL]+LiCl [20 mM] or GTE [100 µg/mL]+CHX [1 µg/mL] or EGCG [100 µM]+CHX [1 µg/mL] or GTE [100 µg/mL]+CHX [1 µg/mL] or MβCD [0.2 mM] or EGCG [100 µM]+MβCD [0.2 mM] or GTE [100 µg/mL]+MβCD [0.2 mM] (6, 12, 24, 48 h) treatment. Membranes were also probed with goat polyclonal anti-actin antibody to normalize protein level. Each experiment was repeated at least three times with similar results. Data are representative values.

EGCG and GTE-induced sClu expression is modulated by various metabolic inhibitors. Western-blot analysis of whole-cell lysates obtained from COLO 205 cells. Immunoblots showing the expression of sClu (40 kDa) and its precursor (60 kDa) form after: (a) H9 [10 µM] or EGCG [100 µM]+H9 [10 µM] or GTE [100 µg/mL]+H9 [10 µM] or PD98059 [50 µM] or EGCG [100 µM]+PD98059 [50 µM]; (b) PMA [100 nM] or EGCG [100 µM]+PMA [100 nM] or GTE [100 µg/mL]+PMA [100 nM] or SB216763 [10 µM] or EGCG [100 µM]+SB216763 [10 µM] or GTE [100 µg/mL]+SB216763 [10 µM]; (c) LiCl [20 mM] or EGCG [100 µM]+LiCl [20 mM] or GTE [100 µg/mL]+LiCl [20 mM] or GTE [100 µg/mL]+CHX [1 µg/mL] or EGCG [100 µM]+CHX [1 µg/mL] or GTE [100 µg/mL]+CHX [1 µg/mL] or MβCD [0.2 mM] or EGCG [100 µM]+MβCD [0.2 mM] or GTE [100 µg/mL]+MβCD [0.2 mM] (6, 12, 24, 48 h) treatment. Membranes were also probed with goat polyclonal anti-actin antibody to normalize protein level. Each experiment was repeated at least three times with similar results. Data are representative values.

or 77.58±3.26 and 71.40±3.17 for EGCG/GTE+CHX [1 µg/mL] or 82.82±8.81 and 77.84±8.50 for EGCG/GTE+MβCD [0.2 mM] treatments.

sClu is under negative-control of β-catenin in COLO 205 cells

Western blot results revealed that in COLO 205 cells EGCG/GTE-induced sClu expression is predominantly controlled by lipid rafts formation, PKC and GSK-3β kinases activity and other, unidentified short-lived proteins since their effects were inhibited by chronic treatment with CHX [1 µg/mL]. Taken these data together, we anticipated a common link represented by β-catenin, as transcriptional activity of this protein is known to be under PKC and GSK-3β negative control (25). PKC could be activated by lipid-rafts-localized 67 kDa laminin receptor, known target of EGCG (26). Finally, β-catenin in free form is also a short-lived protein, thus the presence of translation inhibitor - CHX could significantly affect its action (27). To verify our assumption, we examined the level of nuclear β-catenin after 1 and 2 hour-treatment with EGCG [100 µM] or EGCG+LiCl [20 mM] or EGCG+STS [1 µM] or EGCG+CHX [1 µg/mL] or EGCG+MβCD [0.2 mM]. As shown on Fig. 5B, β-catenin is constitutively active in COLO 205 cells, thus its presence in the nuclei was detected in untreated cells. Furthermore, EGCG treatment slightly decreased β-catenin level, especially at 1st hour of experiment (P<0.001). Conversely, additional presence of STS or MβCD induced β-catenin nuclear translocation (P<0.001)(Fig. 5B, SC). Interestingly, the presence of LiCl, well-known GSK-3β inhibitor, did not modify EGCG-dependent effect on nuclear β-catenin level. Also CHX did not abolish β-catenin nuclear accumulation, β-catenin level was rather constant and at similar level to control cells. Our results indicate that COLO 205 cells stably express nuclear β-catenin even upon STS and MβCD treatment. It correlates with down-regulated expression of sClu.

DISCUSSION

Green tea is a popular beverage, which has received a great deal of attention from researchers and the general public due to its
various possible beneficial effects, such as in the prevention of cancer and cardiovascular diseases (28). In 1987, Yoshizawa et al. (29) first published the inhibitory effects of topically applied EGCG against skin tumor promotion in mice. Thereafter, numerous studies have been conducted, demonstrating the inhibitory activities of tea and tea compounds against tumorigenesis in different models, among them in colon cancer (30, 31). We wondered whether green tea extract (GTE) or/and its main constituent - EGCG are also highly effective in the elimination of metastatic-phenotype cancer cells, represented by human colon adenocarcinoma COLO 205 cell line. Surprisingly, we found that GTE and EGCG time- and dose-dependently stimulated COLO 205 cells survival and proliferation (Fig. 1A, 1C). It was previously reported that GTE/EGCG biological effects are mediated by 67 kDa laminin receptor, which is mainly localized in lipid rafts (13, 32), thus the use of lipid rafts modulators, such as nystatin, imipramine or MβCD was believed by us to reduce GTE/EGCG’s activity (Fig. 1B, 1C). Our observations that lipid rafts modulators inhibit EGCG/GTE-induced effects are in agreement with Fujimura et al. (31), who showed that MβCD treatment limits EGCG-laminin receptor interaction. Further analysis revealed that metabolic activity of COLO 205 cells time-dependently stimulated by GTE/EGCG is accompanied by increased levels of phosphorylated ERK1/2 (Fig. 1E). The phosphorylation level of this MAPK could be inhibited by cholesterol depletion with MβCD (Fig. 2A). The importance of two analyzed signaling pathways, namely PI3K/Akt1/2/GSK-3β and MEK/ERK1/2 in EGCG-mediated stimulatory effects was previously described by Kwon et al. (11) in dermal papillae cells and by Chung et al. (10) in human keratinocytes, both in cell culture models. In contrast, the EGCG-induced activation of ERK1/2 was also reported as a molecular mechanism of inhibition of prostate cancer cells proliferation (33). However, in PC-3 cells the activation of ERK1/2 by EGCG was not inhibited by PD98059 suggesting MEK-independent mechanism of ERK1/2 stimulation. In COLO 205 cells, EGCG-induced ERK1/2 phosphorylation at Y204 residue was abrogated by addition of MEK inhibitor - PD98059 [50 µM]. Consequently, the EGCG/GTE were unable to stimulate cell viability and proliferation. Furthermore, chronic inhibition of ERK1/2 kinase resulted in a dramatic drop of the percentage of viable cells, up to a few percent of initial value (Fig. 2B, 2C). Thus, we assume that MEK-dependent ERK1/2 activity predominantly supports COLO 205 cells survival. To evaluate the role of PI3K/Akt1/2/GSK-3β pathway in EGCG/GTE action the PI3K inhibitor - LY294002 was used. Interestingly, irrespective to the LY294002 concentration we could not reduce P(Ser473)-Akt1/2 phosphorylation as we previously reported (23). The presence of LY294002 could not affect the capability of
EGCG/GTE up-regulate COLO 205 cell survival and proliferation (23). Moreover, we found that in COLO 205 cells upon EGCG/GTE treatment the GSK-3β phosphorylation at Ser9 residue does occur (Fig.1E) unless cholesterol is depleted. Combined treatment with MJβCD and GTE/EGCG caused consistent time-dependent down-regulation of Akt1/2 and GSK-3β levels in the Ser473- and Ser9-phosphorylated form, respectively (Fig. 2A). It could be noticed that MJβCD significantly modulated the Akt/GSK-3β response to GTE/EGCG treatment.

If potent activation of pro-survival signaling pathways occur, one of the identified mechanisms of EGCG positive effect on viability of keratinocytes could be its potency to alter the ratio between the pro-/anti-apoptotic proteins, especially Bcl-2/Bax (10). We examined previously the level of Bcl-2 and Bax proteins, however we did not detect any significant changes in their expression (23). Thus, we focused our attention on clusterin, one of the proteins which was described to be regulated by EGCG/GTE treatment (9).

Whether clusterin (Clu), an enigmatic, multifunctional protein is involved in death or survival of cancer cells is still a matter of debate. Its expression is induced by a wide range of factors, such as cytokines, UV radiation, oxidative stress, changes in calcium homeostasis, as well as some natural compounds, among them EGCG (34-37). Identification of two distinct Clu isoforms: nuclear (nClu) and secretory (sClu) might explain the opposite biological effects induced by Clu (38). It is believed that 50 kDa nClu is shifted from cytoplasm to the nucleus in response to proapoptotic signal. For example, Caporali et al. (9) demonstrated that green tea-extracted catechins were capable of inhibiting the cell growth of prostate cancer SV40-immortalized cells and in particular its nuclear translocation was evident in 17-week old TRAMP, GTE-treated mice model (9). Furthermore, nClu down-regulation or its cytoplasmic retention correlates with the resistance to death signals and is characteristic for cancer cells (9, 39). On the other hand, 80 kDa secretory clusterin (sClu) acts as a chaperone and is proposed to play protective role (40). In our studies, we used monoclonal antibody dedicated for pre-secretory clusterin (60 kDa band) (pre-sClu) and mature 80 kDa secretory clusterin (sClu), but not 50 kDa nClu form. Mature sClu (80 kDa) is a two-subunit protein appearing in western-blot as a 40 kDa band. It should be noted that there are papers where the blots obtained with the same Ab are interpreted as 50 kDa nClu isoform.

Similarly to Caporali et al. (9), we found that EGCG/GTE stimulate sClu expression, however the overall biological effect is quite opposite than in the prostate cancer cells. Further, we found that regulation of sClu expression is complex and apparently could be modulated by the addition of various metabolic inhibitors. The most significant effects on EGCG/GTE-induced sClu levels were demonstrated by PKC kinases inhibitors such as STS and by cholesterol deprivation from lipid rafts with MJβCD (23). Furthermore, EGCG/GTE seems at least in part to rescue the viability of COLO 205 cells from MJβCD treatment. It could result from the release from laminin receptor-associated inhibition of cellular uptake of EGCG/GTE (41), anticipated when cholesterol level dropped. Non lipid rafts-mediated cell signaling pathway seems to be involved in EGCG/GTE effect (Fig. 4D). Potent negative effects were also observed upon lithium chloride-mediated GSK-3β blockage (Fig. 4C) and in CHX co-treated cells (Fig. 4D). To recapitulate the data, we speculate that a common link between the effects evoked by PKC and GSK-3β inhibitors points to redundancy of Wnt signaling pathway, in particular β-catenin transcription factor (42). Wnt canonical pathway is often constitutively active in neoplastic cells, although, normally β-catenin is negatively regulated by GSK-3β that phosphorylates β-catenin to drive it for proteosomal degradation (43). It was demonstrated that PKC kinases are able to differently modulate canonical Wnt pathway. Anyway, β-catenin nuclear accumulation is sustained unless PKC mediates phosphorylation of GSK-3β at Ser9 position. PKC-dependent β-catenin phosphorylation may also tag protein for degradation (44). Noteworthy, PKC activity is often regulated by lipid rafts-anchored G-proteins, thus PKC could be cellular target for EGCG/GTE action (45). Finally, Ju et al. (46) found that oral administration of EGCG resulted in decreased levels of β-catenin in the nuclei. Our western blot results showed that in COLO 205 cells β-catenin is constitutively active and is detected in nuclei of unstimulated cells. In accordance to Ju et al. data (46), EGCG [100 µM] treatment decreased β-catenin nuclear level, whereas additional presence of STS [1 µM] or MJβCD [0.2 mM] resulted in a time-dependent stimulation of nuclear β-catenin translocation. In comparison to clusterin expression, we can easily recognize that in COLO 205 cells clusterin expression is negatively related to β-catenin nuclear levels. Similar conclusions were drawn by Schepler et al. (47) who found that GFP-cyt-E-cadherin-transfected HCT116 colon carcinoma cells show strong decrease in nuclear β-catenin, whereas at the same time the level of 80 kDa sClu was predominantly up-regulated and exhibited cytoplasmic localization.

In conclusion, we have demonstrated that in COLO 205 cells EGCG and GTE stimulate cell survival and proliferation in a lipid rafts-dependent manner via at least MEK/ERK1/2- but not Akt1/2/GSK-3β-dependent manner. Furthermore, EGCG/GTE-mediated positive effects on viability and mitogenicity of COLO 205 even during suppression of β-catenin transcriptional activity, which was correlated with sClu clusterin expression. Bearing in mind the potent protective effects of secreted clusterin, the results published by Golden et al. (15), concerning the negative effect of green tea catechins upon effectiveness of chemotherapy seems rational. We suggest that in analyzed patients, EGCG/GTE-induced sClu expression was associated with ordinary sticking to chemotherapeutics, thus preventing their action on the intact cell membrane.

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