Green Tea Extract Modulates Actin Remodeling via Rho Activity in an In vitro Multistep Carcinogenic Model

Qing-Yi Lu,1 Yu-Sheng Jin,2 Allan Pantuck,3 Zuo-Feng Zhang,4 David Heber,1 Arie Belldegrun,3 Mai Brooks,5 Robert Figlin,6 and JianYu Rao2

1Center for Human Nutrition and Departments of 2Pathology and Laboratory Medicine, 3Urology, 4Epidemiology, 5Surgery, and 6Medicine, University of California at Los Angeles, Los Angeles, California

ABSTRACT

Alteration of actin polymerization and loss of actin filaments is a marker of cellular dedifferentiation and early malignant transformation. To study this phenomenon, an in vitro urothelial model consisting of two cell lines, HUC-PC and MC-T11, were incorporated into the study design. These two cell lines have different malignant transformation potential. The effect of green tea extract (GTE), a potential anticancer agent, on actin remodeling was investigated. Upon exposure to the carcinogen 4-aminobiphenyl (4-ABP), the untransformed HUC-PC undergoes malignant transformation whereas the transformed MC-T11 progresses from noninvasive to invasive tumor. GTE induces actin polymerization in MC-T11 cells in a dose-responsive manner, but this effect is less obvious in the untransformed, more differentiated HUC-PC cells, which natively have higher actin polymerization status. In contrast, GTE antagonizes carcinogen 4-ABP induced actin depolymerization and stress fiber disruption in HUC-PC cells. In MC-T11 cells, GTE inhibits 4-ABP induced motility by increasing cell adhesion and focal adhesion complex formation. The effect of GTE on actin remodeling seems to be mediated by the stimulation of small GTP-binding protein Rho activity, because C3 exoenzyme, a specific inhibitor for Rho, blocks GTE-mediated Rho activation and stress fiber formation in MC-T11 cells. This study shows that GTE exerts an effect on cytoskeletal actin remodeling and provides further support for the use of GTE as a chemopreventive agent.

INTRODUCTION

Tea is one of the most widely consumed beverages in the world (1). Numerous epidemiologic and animal studies have suggested that green tea extract (GTE) provides strong anticancer effects for a number of human cancers, including bladder cancer (2, 3). At the laboratory level, a variety of biological effects of GTE including antiproliferation, antiangiogenesis, and apoptosis induction etc., have been observed in various in vitro cell models (4, 5). Recent studies have shown that tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) strongly inhibits DNA methyltransferase and reactivates methylation-silenced tumor suppressor genes in cancer cell lines (6) suggesting that epigenetic mechanisms play an important role in GTE-associated anticancer activities. However, the exact biochemical and molecular pathways leading to the anticancer effects of GTE are still not fully understood. In addition, it is unclear whether the anticancer effect of GTE is more preventive or more therapeutic. A recent phase II trial showed a negative effect of GTE in patients with androgen-independent metastatic prostatic cancer (7) suggesting that GTE may have limited therapeutic effect in the late-stage cancers.

This study has been done to test the chemopreventive effect of GTE on an in vitro urothelial model, using cytoskeletal actin remodeling as a surrogate marker. Cytoskeletal actin is a ubiquitous protein present in all eukaryotic cells (8). As one of the major proteins in the cell, actin and its associated proteins play important structural and functional roles, such as maintaining cell morphology, cell adhesion, cell motility, exocytosis and endocytosis, and cell division (9–14). Accumulated evidence suggests that alteration of actin remodeling is a common effector event linking the signal transduction activities of several important oncogene families, the most notable one being the Ras superfamily GTPase Rac, Rho, and CDC42, to malignant cellular phenotypes, including alteration of morphology, increased motility, and angiogenesis etc. (for review, see ref. 15). However, exactly how the actin network is altered in the malignant transformation and progression processes, as well as the underlying mechanisms of alteration, are far from clear. Our previous studies have shown that actin polymerization status, manifested as the ratio of filamentous (F) to globular (G)-actin (F/G ratio), is a generalized marker of cellular differentiation (16). Disrupted actin stress fibers and actin depolymerization with shifting of F-actin to G-actin occurs in early stage of malignant transformation, which can serve as a marker to assess bladder cancer risk (17, 18). This marker represents bladder cancer associated “field” defect, and more importantly, correction of such “field” defect by intravesical application of cell differentiation agent DMSO in patients who received transurethral resection prevents bladder cancer recurrence (18, 19). These findings suggest that actin remodeling may serve as a target, and modulating cellular actin status may provide a surrogate marker for bladder cancer chemoprevention trial.

Recently, with the support of National Cancer Institute, a chemoprevention trial using GTE as one of the treatments has been initiated in our group with the ultimate goal of preventing bladder cancer recurrence in bladder cancer patients who are former smokers. The goal of our current study is to determine
whether GTE modulates cellular actin remodeling during the urothelial transformation process, and if so, by what mechanisms. A unique in vitro bladder cancer carcinogenesis model is used. The model consists of two cell lines derived from the same normal human urothelial clone, immortalized by SV40 transfection (20). The biochemical and molecular characteristics including cytogenetic changes of these cell lines have been well characterized previously (20). The HUC-PC cell line is an untransformed cell line that does not form tumor nodules when injected into nude mice, whereas MC-T11 is a transformed line. However, by exposure to the carcinogen 4-aminobiphenyl (4-ABP), HUC-PC cells undergo malignant transformation and MC-T11 cells progress from low-grade noninvasive tumors to high-grade invasive tumors (21). Thus, this model simulates the early stages of bladder cancer carcinogenesis. Our study shows for the first time that GTE has substantial effect on modulating actin remodeling in urothelial cells, which are associated with increased cellular adhesion and inhibition of motility. This effect seems to be mediated, at least partially, through the stimulation of Rho activity.

MATERIALS AND METHODS

Materials. GTE was obtained from Pharmanex, Inc. (Provo, UT) and 4-ABP was purchased from Sigma Chemical Co. (St. Louis, MO). The purity of the catechins in the GTE was 84%. The Pharmanex GTE is a mixture of many catechin compounds, with EGCG as a major component (43.0% by weight), followed by epicatechin-3-gallate (13.7%), epicatechin (6.0%), galloctechnin gallate (5.6%), epigallocatechin (4.0%), galloctechnin (2.3%), catechin (2.0%), catechin gallate (1.4%). In this study, the concentration was expressed as the amount of GTE per milliliter of media bathing the cells (μg/mL).

Cell Culture. Both HUC-PC and MC-T11 cells were grown in 90% F-12 nutrient mixture (Ham) medium (Life Technologies, Grand Island, NY) with 1% penicillin, 10 mg/mL streptomycin, and 10% fetal bovine serum. Cultures were maintained at 37°C in 5% CO2 and 95% air, and medium changed twice per week. GTE and its catechins were dissolved in double-distilled H2O to make a stock solution of 10 mg/mL. 4-ABP was dissolved in 100% DMSO to make a stock solution of 100 mM.

Logarithmically growing HUC-PC and MC-T11 cells were harvested and seeded at an initial density of 2 × 10⁶ cells in 5 mL of fresh medium in 60-mm Petri dishes. After overnight proliferation, the adherent cells at a concentration of 1 × 10⁵ cells/mL were incubated with GTE at final concentrations of 20, 40, and 80 μg/mL at different time points (12, 24, and 48 hours), or with 4-ABP (200 μmol/L) at first for 12 hours, followed by various concentrations of GTE for additional 24 hours, or a combination of GTE and 4-ABP simultaneously. The final solvent concentrations in the medium were <0.1% (v/v), which had minimum effect on the cells. At the end of each treatment, cells were harvested and centrifuged at 500 × g for 5 minutes. The total number of viable cells for each condition was determined by trypan blue exclusion test.

DNase I Inhibition Assay. The previously described DNase I inhibition method was used without modification for G-actin and total actin quantification (21). The quantity of F-actin was derived by subtracting G-actin from total actin and thereby the ratio of F-actin to G-actin was determined.

Fluorescence Analysis. For fluorescence analysis, cells cultured directly on 1-cm-diameter cover glass were fixed with 3.7% paraformaldehyde for 30 minutes and subjected for triple labeling of either F-actin/G-actin/DNA or F-actin/paxillin/DNA. For the former, cells were incubated sequentially with 1:150 Alexa Fluor 647-conjugated DNase I (Molecular Probes, Inc., Eugene, OR) for 30 minutes, 100 μL of Bodipy conjugated Phalloidin (Molecular Probes; 1:200 v/v) for another 30 minutes, and 1:1,000 dilution of 4’,6-diamidino-2-phenylindole (10 mg/mL, Molecular Probes) for 5 minutes. For the latter, cells were incubated with 1:100 monoclonal anti-Paxillin (clone 5H11, Upstate Biotechnology, Lake Placid, NY) for 1 hour, 1:150 Cy5-conjugated AffiniPure Goat Anti-Mouse IgG (H+L; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 minutes, 1:40 Bodipy phallacidin (for F-actin; Molecular Probes) for 30 minutes, and 1:1,000 dilution of 4’,6-diamidino-2-phenylindole for 5 minutes. Between each incubation step, the cover glass was rinsed with PBS thrice. The stained cover glass was then transferred onto a regular microscopic slide, which was then mounted in 100 mM n-propyl gallate (Sigma Chemical) in spectranalyzed glycerol (Fisher Scientific, Pittsburgh, PA), pH 6.5, for fluorescence microscopic examination. Images were generated using a Nikon TE300 microscope equipped with an Imaging Microimager II digital camera.

Cell Adhesion and Migration Assays. For adhesion analysis, 96-well tissue culture plates coated with 50 μg/mL fibronectin for 1 hour at 37°C were used. After blocking with 1% bovine serum albumin for 30 minutes, plates were seeded at a concentration of 5 × 10⁶ cells per well with untreated cells and cells treated with GTE, 4-ABP, or 4-ABP combined with GTE at different time points (30 minutes, 1, 3, 12, and 24 hours). After washing off the nonattached cells with 1× PBS and shaking, the remaining attached cells were fixed in 1% glutaraldehyde for 15 minutes, and stained with 0.1% (w/v) crystal violet for 20 minutes. Cells were lysed in 1% SDS. The intensity of stain, in direct proportion to the number of adherent cells, was quantitated by absorbance at 540 nm using a microplate reader. Each cell line was tested in three separate wells over three independent experiments. For migration assay, a uniform cell-free area was created by scratching confluent monolayers with a plastic pipette tip, and the wound area was inspected at different time intervals to determine the distance migrated by the cells. The closer the gap, the faster the cell migrates. At each time point, four photographs were taken and the number of cells/mm² migrated into the area of wound was counted. For both assays, results are expressed at mean ± SD of three independent experiments.

Immunoblot Analysis. Cells that were washed twice in cold PBS were scraped from culture dishes in lysis buffer [50 mMol/L Tris-HCl (pH 7.4), 150 mMol/L NaCl, 2 mMol/L EGTA, 2 mMol/L MgCl2, 1% (v/v) Triton X-100, 10% glycerol, 10 mMol/L DTT, 1 mMol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotenin, 5 μg/mL pepstatin A, 50 mMol/L NaF, and 1 mMol/L Na3VO4]. Lysates were centrifuged at 12,000 × g and 4°C for 10 minutes. Protein concentrations of lysates were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). For Immunoblot analyses, the same amount of proteins (30 or 50 μg) were
subjected to 8% SDS-PAGE and were electrotransferred to nitrocellulose membranes using electroblot buffers. Membranes were blocked in PBS containing 5% nonfat dry milk for 30 minutes. Reactions with the primary antibodies in TBS buffer containing 3% dry milk were carried out at 4°C overnight. After extensive washing, membranes were placed on a shaker with biotinylated secondary IgG for 1 hour. Upon further washing, membranes were reacted with streptavidin-horseradish peroxidase for 45 minutes and enhanced chemiluminescence detection reagents immediately before autoradiography.

Rho Activation Assay and C3 Exoenzyme Inhibition Assay. The exact protocol from the manufacter’s instructions was followed (Upstate Biotechnology). Briefly, activated, GTP-bound Rho proteins were isolated by coprecipitation with the Rho-binding domain of rhodekin, and the total as well as activated Rho were then detected by Western blot. Positive (GTPγS) and negative (GDP) controls were loaded on the gel for each assay. To inhibit Rho activity, inhibitor C3 exoenzyme from Clostridium botulinum (Calbiochem, San Diego, CA) was introduced into cells before initiating the experiments. To do so, 5 μg/mL C3 exoenzyme was mixed with 5 μg/mL lipofectin (Invitrogen, Carlsbad, CA) 1:1 in medium and incubated with MC-T11 cells for 12 hours at 37°C.

Statistical Analysis. Descriptive statistics, such as mean and SD, were used to summarize the results. Student’s t tests were used for univariate analysis. Statistical significance was defined by a two-tailed P = 0.05.

RESULTS

Green Tea Extract Induces Actin Polymerization in Transformed MC-T11 Cells. We first examined the effect of GTE on actin polymerization, which was both quantitatively measured by biochemical DNase I inhibition assay and morphologically by fluorescence microscopic analysis. Dosage levels of 20 to 80 μg/mL were tested because previous experiments showed that GTE exhibited strong tumor inhibitory effect at these concentrations in a mouse xenograft (22). In the transformed MC-T11 cells, after 24 hours of incubation of GTE, a dose-response increase of actin polymerization, as reflected by an increased FG-RI (F/G-actin ratio increment relative to untreated control), which was determined by DNase I inhibition assay, was observed (Fig. 1A). The FG-RI increased to 24%, 33%, and 45% over the untreated control with 20, 40, and 80 μg/mL GTE, respectively. The stimulation effect of GTE on actin polymerization was confirmed morphologically by fluorescence microscopic analysis, in that GTE-treated MC-T11 cells (40 μg/mL) had increased green fluorescence (F-actin) and stress fiber formation and concomitantly decreased G-actin (red fluorescence) compared with the untreated control (Fig. 1B). However, the effect of GTE on HUC-PC cells, the untransformed cells with a natively high level of actin polymerization status, was much less obvious (Fig. 1A and B). Thus, it seems that GTE has a selective effect on stimulating actin polymerization in the transformed MC-T11 cells over the untransformed HUC-PC cells.

We then tested eight different catechins for their effect on actin polymerization in MC-T11 cells (Fig. 1C). A similar finding as described above was seen in several catechins.

Fig 1  Effects of GTE on actin polymerization in MC-T11 and HUC-PC cells. A, dose-response effect of GTE on FG-RI. MC-T11 and HUC-PC cells treated with various concentrations (20, 40, 80 μg/mL) of GTE for 24 hours were harvested, and the FG-RI were determined by DNase I inhibition assay as described in Materials and Methods. FG-RI was calculated as the % increase of F/G ratio in treated sample versus the parallel-untreated control sample (i.e., [sample F/G ratio – control F/G ratio] / control F/G ratio × 100%). Columns, mean of three independent experiments; bars, ±SD. B, fluorescence morphological analysis of F-actin versus G-actin. HUC-PC and MC-T11 cells treated with or without (ctrl) 40 μg/mL GTE for 24 hours were washed, fixed, and labeled sequentially for F-actin (green fluorescence), G-actin (red fluorescence), and DNA (blue fluorescence), as detailed in Materials and Methods. Note the untransformed HUC-PC cells had higher level of F-actin even in the untreated (ctrl) cells and had little change of actin staining patterns in response to GTE. In contrast in MC-T11 cells, GTE increased F-actin stress fiber formation, with concomitant decrease of G-actin. Images were taking using a Nikon Eclipse E400 microscope at 20× object. C, dose-response effect of eight different catechins GTE on FG-RI.
including catechin, EGCG, catechin gallate, and galotechin; however, none of the individual catechins had as strong effect as GTE itself for stimulating actin polymerization. Thus, we used GTE itself, rather than individual catechins, for the remaining experiments.

**Green Tea Extract Induces Actin Polymerization in Carcinogen 4-Aminobiphenyl-Treated HUC-PC Cells and Antagonizes Cytochalasin E–Induced Actin Depolymerization in Both Cells Lines.** Previously, we reported that the carcinogen 4-ABP induced actin depolymerization in the untransformed HUC-PC cells, and that the depolymerization of actin was correlated with an increased risk of malignant transformation as shown by tumor formation when treated cells were inoculated in nude mice (20, 21). In this experiment, we tested whether GTE had any effect on 4-ABP-treated HUC-PC cells. HUC-PC cells first exposed to 4-ABP (200 μmol/L) for 12 hours were further incubated with or without GTE (40 μg/mL) for additional 24 hours. Consistent with previously reported findings, HUC-PC cells treated with 4-ABP alone for 36 hours had an increased G-actin level, a decreased F-actin level, and a decreased F/G-actin ratio compared with the untreated control, indicating actin depolymerization following 4-ABP exposure (Fig. 2A). However, when cells were treated with 4-ABP first for 12 hours, followed by GTE for additional 24 hours, the F-actin level and F/G-actin ratio were increased whereas G-actin level decreased compared with cells treated with 4-ABP alone. These levels almost reached the levels of untreated control. Again, morphologically, 4-ABP alone decreased actin fibers (F-actin, 

**Fig. 2 GTE increased actin polymerization in 4-ABP treated HUC-PC cells (A) and restores actin polymerization in cytochalasin E (CE) treated HUC-PC and MC-T11 cells (B). A, HUC-PC cells were cultured under each of following conditions: solvent control, 4-ABP (200 μmol/mL) only for 36 hours, GTE only (40 μg/mL) for 36 hours, and 4-ABP (200 μmol/L) for 12 hours followed by adding GTE (40 μg/mL) for 24 hours. F-actin, G-actin, and F/G-actin ratio were determined by DNase I inhibition assay as described in Materials and Methods. For G-actin and F-actin levels, values were expressed as μg per 10⁶ cells, and F/G-actin ratio was expressed as the original value × 10. Note that 4-ABP decreased F-actin, increased G-actin, hence decreased F/G-actin ratio, whereas GTE restored actin polymerization with increased F-actin and F/G-actin ratio, and decreased G-actin, in cells preexposed to 4-ABP. Columns, mean of three independent experiments; bars, ±SD. For morphology, cells were processed as described in Fig. 1B and images were taken at 60× object. B, HUC-PC and MC-T11 cells were cultured under each of following conditions: solvent control, 20 nmol/L CE only for 48 hours and 20 nmol/L CE for 24 hours followed by adding GTE (40 μg/mL) for 24 hours. Cells were processed and fluorescence was labeled as described in Fig. 1B and images were taken at 20× object.**
green), whereas GTE partially restored F-actin fibers with a concomitant decrease of G-actin (red fluorescence; Fig. 2A). This finding suggests that GTE has an antagonizing effect on 4-ABP-induced actin depolymerization. Exact same results were obtained when GTE and 4-ABP were added simultaneously (data not shown).

To further characterize how GTE affects actin polymerization process, we tested the effect of GTE on cytochalasin E–treated MC-T11 and HUC-PC cells. Cytochalasin E is a potent actin depolymerization agent that binds and caps the barbed end of actin filaments to prevent actin elongation. Cells treated with cytochalasin E (20 nmol/L) for 24 hours were aliquot into two culture conditions, one with and one without GTE (40 μg/mL). Both HUC-PC and MC-T11 cells, when exposed to cytochalasin E for a total of 48 hours, showed marked actin depolymerization with decreased F-actin (green fluorescence) as well as total loss of stress fibers, and simultaneously increased G-actin (red fluorescence; Fig. 2B). However, the addition of GTE to the cell culture preexposed to cytochalasin E resulted in an increase in F-actin (green fluorescence) and decrease in G-actin (red fluorescence), and the restoration of stress fibers (Fig. 2B). This finding suggests

Fig. 3  Effects of GTE on cell adhesion (A), motility (B), and adhesion complex formation (C). In these experiments, cells were cultured with solvent control, GTE alone (40 μg/mL), 4-ABP alone (200 μmol/L), or GTE (40 μg/mL) plus 4-ABP (200 μmol/L). For adhesion analysis (A), cells growing on fibronectin-coated culture plates at specified time points were fixed with glutaraldehyde, stained with crystal violet, and measured by a microplate reader, as detailed in Materials and Methods. Columns, mean of three independent experiments; bars, ±SD. Note that after 12 hours of treatment, 4-ABP decreased adhesion significantly (+, P < 0.05; Student’s t test) and GTE increased adhesion significantly (*, P < 0.05; Student’s t test). After 24 hours of incubation, cell adhesion was significantly increased in GTE + 4-ABP–treated cells compared with 4-ABP alone treated cells (#, P < 0.05; Student’s t test). For migration assay (B), wound was created by a micropipette tip as described in Materials and Methods, and no. cells/mm² migrated into the area of wound at different time point was counted. Columns, mean of three independent experiments; bars, ±SD. The accompanied photos showed F-actin staining of the area of wound after 12 hours of wound creation, whereas areas between the solid lines represented the distance of original wound at time 0. Photos were taken at 10× object. The increased adhesion and decreased motility in GTE alone or GTE + 4-ABP–treated cells correlated with the increased stress fibers and cell adhesion complex formation, as in C of the triple labeling of F-actin (green fluorescence), paxillin (red), and DNA (blue). Note that 4-ABP alone–treated cells had decreased stress fibers and polarized distribution of adhesion complex (white arrowhead), whereas GTE alone– or GTE + 4-ABP–treated cells had increased stress fiber and focal complex formation. The focal complex formation was distributed in the entire cell periphery. Images were taken at 100× object under oil immersion.
that GTE directly modulates the actin polymerization process, which antagonizes cytochalasin E–induced actin depolymerization unselectively in both cell lines.

**Green Tea Extract–Induced Actin Polymerization Correlates with Increased Cell Adhesion/Attachment and Decreased Motility in MC-T11 Cells.** Previously, Bookland et al. reported that when the carcinogen 4-ABP-treated MC-T11 cells were injected into nude mice, the mice formed highly invasive tumors, whereas the untreated control cells developed only noninvasive tumors (20). This finding suggests that 4-ABP promotes the progression of MC-T11 cells from noninvasive phenotype to invasive phenotype. Because actin plays an important role in tumor cell motility and presumably tumor cell invasion, we hypothesize that actin remodeling may be involved in this process. To determine how actin was changed in MC-T11 cells in response to 4-ABP treatment, and whether GTE had any effect on 4-ABP-treated MC-T11 cells, we compared 4-ABP alone–treated cells and cells treated with 4-ABP and GTE together on actin morphology, cell adhesion, and motility. Figure 3A shows the time course effect of GTE and 4-ABP on cell adhesion in MC-T11 cells on a fibronectin-coated surface. The degree of cell adhesion was increased with time. However, after 12 hours of treatment, GTE (40 μg/mL) significantly enhanced cell adhesion relative to control, and 4-ABP significantly inhibited cell adhesion. In cells exposed to 4-ABP (200 μmol/L) combined with GTE (40 μg/mL), GTE partially restored cell adhesion after 24 hours of incubation. The increased cell adhesion correlated with decreased cell motility, as shown in Fig. 3B. With the wound-scratching assay, the number of cells migrated into the wound area (an indication of motility) was notably different after 6 hours’ incubation of 4-ABP and GTE, whereas GTE inhibited and 4-ABP enhanced cell migration compared with untreated control. However, the increased cell migration was suppressed when cells were coincubated with GTE and 4-ABP simultaneously for 12 hours.

Morphologically, we observed that the increased cell adhesion and decreased cell motility in cells treated with GTE were accompanied by an increased actin stress fiber and focal adhesion complex formation, which was determined by a triple-immunofluorescence labeling of F-actin, paxillin (a marker of cell adhesion complex), and DNA (Fig. 3C). Compared with untreated control cells, as shown in Fig. 3C, cells exposure to GTE (40 μg/mL) for 24 hours showed markedly increased stress fibers (F-actin, green fluorescence) as well as focal adhesion complex formation (paxillin, red fluorescence). The adhesion complex was distributed at the entire periphery of cells in a circumferential manner, which could explain the decreased motility of GTE-treated cells. In contrast, 4-ABP alone–treated cells showed a more elongated shape with overall a decreased focal adhesion complex and a polarized distribution of adhesion complexes at the two ends (white arrowhead). As expected, coincubation of 4-ABP–treated cells with GTE simultaneously restored the focal stress fiber as well as adhesion complex formations in a circumferential distribution. It should be noted that similar effect of GTE on cell adhesion, motility, and actin stress fiber formation was observed when cells were exposed to 4-ABP first, followed by GTE (data not shown).

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**Green Tea Extract Induces Actin Polymerization and Stress Fiber Formation via Stimulating Rho Activity.** In an attempt to determine the potential mechanisms of the effect of GTE on actin polymerization, cell adhesion and migration, we first examined focal adhesion kinase (FAK) activities, because FAK plays important role in regulating focal adhesion complex formation, which may also lead to alterations of actin polymerization and stress fiber formation. The levels of phospho-FAK and total FAK were examined by immunoblot in MC-T11 cells treated with GTE (40 μg/mL) and untreated control. No significant changes of the expression were seen for either phospho-FAK or total FAK (Fig. 4A). The expression of...
paxillin itself, an important component of cell adhesion complex, was also unchanged (Fig. 4A).

In contrast, a marked increase in the activity of Rho, a regulator of actin stress fiber formation, was detected using a rhotecin-based pulldown assay. The increased Rho activity was observed 20 minutes after GTE incubation in MC-T11 cells. The activity was further increased at 2 hours (Fig. 4B). Cells preloaded with C3 exoenzyme for 12 hours, a specific Rho activity inhibitor, by ADP ribosylation using liposomal delivery blocked the GTE induced stress fiber and focal complex formations (Fig. 4C). Therefore, the capability of GTE in actin remodeling seems to be mediated, at least partially, by the stimulation of Rho activity.

DISCUSSION

Whereas numerous anticancer activities have been reported for GTE, our study, for the first time, shows that GTE directly modulates actin polymerization and distribution, thus enhances cell adhesion and inhibits motility. GTE also directly antagonizes the actin depolymerization effect of 4-ABP, thereby inhibited 4-ABP induced cell motility. This is significant because microfilament actin plays many functional roles in cells, including maintaining cellular morphology, cell adhesion and motility, cell cycle control, and cell death machinery, among many others (9, 10). Extensive studies have shown that actin filaments are regulated by actin signaling proteins that are components of important oncogenic signal transduction pathways, the most notable candidate being the small GTGase of Ras superfamily proteins Rac, Rho, and Cdc42 (12). In addition, a large number of actin-binding proteins have been cloned, many of these proteins have tumor-suppressive functions and are invariably involved in malignant transformation process (for review, see ref. 15). Because malignant cells often manifest with dramatic changes of many, if not all, of the above mentioned features (altered morphology, loss of cell adhesion, and increased invasiveness, etc.), it has been postulated that actin may function as an important mediator between oncogenic signal transduction activities and malignant phenotypes. Thus, our findings may provide another potential mechanism to explain the anticancer effect of GTE.

As indicated in Materials and Methods, the Pharmanex GTE used in this study is a mixture of many catechin compounds, with EGCG as a major component (43.0% by weight). This is similar to polyphenon E used in the National Cancer Institute–supported trial. The polyphenon E contains 80% to 98% total catechins by weight; the main component is EGCG, which comprises 50% to 75% of the material. Other catechins are present in levels ranging from ~2% to 12% each, including epicatechin, epigallocatechin, epicatechin-3-gallate, and gallatechin gallate (23). In this in vitro study, three concentration levels were examined in the cell lines (20, 40, and 80 μg/mL). These concentrations were selected based on the data from an in vivo animal study. In that study, a maximum tumor inhibition effect of GTE was observed at 40 μg/mL (22). This concentration, although may be higher than expected concentrations achieved in the clinical trial (800 mg daily), is compatible with most in vitro studies reported in the literature (usually around 0-40 μg/mL; refs. 2, 4, 5).

This study has several limitations. First of all, whereas this study showed an antagonistic effect of GTE on carcinogen 4-ABP on actin depolymerization in the HUC model, the exact mechanism of how 4-ABP alters actin remodeling is not yet determined. It is also undetermined specifically how 4-ABP induced transformation in HUC-PC cells and tumor progression in MC-T11 cells, although the mutagenic effect of 4-ABP is presumably involved (24). However, according to Bookland et al., mutations of ras oncogene does not seem to be involved in 4-ABP induced tumorigenecity in these cell lines (24). We reported previously that the malignant transformation and progression seems to be associated with progressive alteration of actin dynamics from cytoplasm to nuclear (21). Thus, we hypothesize that GTE may function as a down stream effect of 4-ABP induced pathways that lead to alteration of actin remodeling. However, exact how this occurs remains to be determined. Another potential limitation of the current study is that a crude GTE instead of individual catechines was used in most of the experiments, which may result in a pleiotropic effect. We did test eight different catechines in our preliminary study, and four of eight showed actin polymerization effect but none as strong as GTE itself, suggestive a potential synergistic effect of these chemicals on actin remodeling.

Our current study shows that the effect of GTE on actin remodeling seems to be mediated, at least partially, by stimulating Rho activity; because a specific Rho inhibitor, the C3 exoenzyme, could block such an effect. This observation, however, does not exclude other potential mechanisms, especially in view of the fact that the main GTE components, EGCG, poses strong inhibitory effect on DNA methyltransferase (6). Because many of the actin binding and regulating proteins, such as gelsolin, an actin-capping and actin-severing protein, have CpG-rich regions and are prone to epigenetic regulation (25–29), it is possible that part of the effect of GTE on actin remodeling may be mediated by altered expression of these protein targets. Thus, further investigations aimed at examining many of these targets simultaneously may be worthwhile.

It should be emphasized that actin remodeling as well as the associated cell adhesion and migration is a complex and carefully orchestrated cellular process that involves many signal transduction events as well as actin binding and regulatory proteins, as mentioned above. Adhesion-related processes seem to be broadly rate limiting for the movement of epithelial cells and fibroblasts, and maximum speed occurring at an intermediate adhesiveness, because at high adhesiveness, cells are unable to break attachments (30). Distribution of adhesion complex is also important, as McHardy et al. showed previously that a specific tumor invasive inhibitor, dihydroxymuroporamic C, induces more stress fiber and focal adhesion complex formations, and a round cell morphology, rather than the point end of elongated cells (31). Interestingly, the effect of dihydroxymuroporamic C on actin polymerization is also mediated by Rho (31), a finding that is similar to GTE as we report herein.

Recently, great efforts have been initiated to develop so called “Intelligent Drugs” that specifically target the Ras GTGase superfamily proteins, especially those involved in actin dynamics such as Rac, Rho, and CDC 42 (for review, see ref. 15). This strategy is based on the assumption that tumor cells may
have increased activities of some of these proteins (32). Although not consistent, some studies show that expressions of these proteins, such as Rho, may be elevated in late-stage metastatic cancer cells (33). It should be noted that such observation is not entirely contradictory to our current observation, because it is possible that the actin dynamics might be different in the late-stage metastatic cancer compared with early stages of bladder carcinogenesis, as shown previously (34). For instance, typically in the early stage of malignant transformation, such as at the stage of intraepithelial neoplasia, the tumor cells are characterized by an increased nuclear/cytoplasmic ratio and a decreased cytoplasmic actin fibers (35). This is the stage in which chemoprevention strategy is most likely to be effective. At the later stage, however, tumor cells may have different actin remodeling pattern that may be the result of, or associated with tumor cell’s overall genetic instability (21). Such actin patterns may be the driven force for tumor cell aggressiveness such as invasion and metastasis. It will be interesting, therefore, to determine whether such different patterns of actin dynamics may somehow associate with the negative effect observed in the GTE trial for late-stage prostate cancer (7). Regardless, our finding that GTE stimulates Rho activity and the fact that some Rho isoforms (e.g., RhoB) actually function as a tumor suppressor (36), suggest that caution should be taken in the design of the trial involving the small molecules. Our data also suggest that Rho activity may be used to monitor the effects of GTE/polyphenol E in clinical trials. It may be worthwhile to study whether and how the down stream signaling events such as mitogen-activated protein kinases are altered in response to GTE, and whether such events can be the targets of chemoprevention agents.

In summary, this study shows that under in vitro conditions GTE induces actin polymerization in transformed MC-T11 cells, antagonizes carcinogen 4-ABP-induced actin depolymerization in untransformed HUC-PC cells, and inhibits 4-ABP-induced motility in transformed MC-T11 cells. These findings further support the use of GTE as a chemopreventive agent for bladder cancer. However, additional studies on the mechanisms of GTE-induced Rho activation and correlation with tumor phenotype, such as invasion and metastasizes in vivo will be necessary to fully elucidate the mechanisms as well as the associated biological alterations.

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Green Tea Extract Modulates Actin Remodeling via Rho Activity in an *In vitro* Multistep Carcinogenic Model


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