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Mechanism-based inhibition of cancer metastasis with (−)-epigallocatechin gallate

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ABSTRACT

Cell motility and cell stiffness are closely related to metastatic activity of cancer cells. (−)-Epigallocatechin gallate (EGCG) has been shown to inhibit spontaneous metastasis of melanoma cell line into the lungs of mice, so we studied the effects of EGCG on cell motility, cell stiffness, and expression of vimentin and Slug, which are molecular phenotypes of epithelial–mesenchymal transition (EMT). Treatments of human non-small cell lung cancer cell lines H1299 and Lu99 with 50 and 100 μM EGCG reduced cell motility to 67.5% and 43.7% in H1299, and 71.7% and 31.5% in Lu99, respectively in *in vitro* wound healing assay. Studies on cell stiffness using atomic force microscope (AFM) revealed that treatment with 50 μM EGCG increased Young's modulus of H1299 from 1.24 to 2.25 kPa and that of Lu99 from 1.29 to 2.28 kPa, showing a 2-fold increase in cell stiffness, i.e. rigid elasticity of cell membrane. Furthermore, treatment with 50 μM EGCG inhibited high expression of vimentin and Slug in the cells at a leading edge of scratch. Methyl-β-cyclodextrin, a reagent to deplete cholesterol in plasma membrane, showed inhibition of EMT phenotypes similar that by EGCG, suggesting that EGCG induces inhibition of EMT phenotypes by alteration of membrane organization.

1. Introduction

Cancer metastasis is a complex condition: Patients are sometimes aware of metastasis much earlier than primary cancer, especially with brain metastasis of lung cancer and bone metastasis of prostate cancer. Thus it is essential to know about preventing metastasis even before clinical onset of primary cancer. Before cancer preventive activity of green tea catechins was proved in humans [1,2], we previously demonstrated that peroral administration of 0.05–0.1% (−)-epigallocatechin gallate (EGCG) in drinking water significantly inhibited spontaneous lung metastasis of B16-BL6 cells inoculated into the right foot pad of male C57BL/6 mice [3]. Among numerous cancer preventive functions of EGCG and green tea catechins, Gimzewski's group was the first to report that green tea extract increased the stiffness of body fluid cancer cells obtained from human cancer patients, indicating that EGCG causes a significant increase in the membrane elasticity of cancer cells [4]. In light of this evidence, we first studied the effects of EGCG on the metastatic potential of highly metastatic human lung cancer cell lines H1299 and Lu99 in *in vitro* wound healing assay, and on cell stiffness measured by atomic force microscope (AFM). AFM provides Young's modulus: Highly metastatic cancer cells show low Young's modulus, indicating lower cell stiffness (softer elasticity) with higher cell motility [5–7]. Our experiments showed that treatment with EGCG dose-dependently inhibited cell motility and increased Young's moduli of H1299 and Lu99 cells, resulting in higher cell stiffness and rigid elasticity. Cell motility and cell stiffness are now recognized as mechanical phenotypes of epithelial–mesenchymal transition (EMT): EMT is theoretically understood as acquisition of the phenotypes of mesenchymal cells, such as fibroblasts, by epithelial cells, and cancer cells undergo a partial or complete EMT, resulting in increase in cell motility and invasiveness [8,9]. Thus, lung cancer cell lines with mesenchymal status show lower Young's modulus than cell lines with epithelial status [10,11]. During the EMT process, numerous molecular processes are engaged, including activation of transcription factors, expression of mesenchymal proteins and reorganization of cytoskeletal proteins [8,9,12]. Activation of EMT programs is stimulated by various biological factors, such as tumor growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α), and also by mechanical stimuli, such as disruption of cell–cell adherent junction and stiffness of extracellular matrix (ECM) in the tumor

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microenvironment [8,9,12,13]. In addition, mechanical stimuli induced the expression of mesenchymal proteins (vimentin and N-cadherin) and transcription factors (Slug) in a subpopulation of cells at a leading edge of scratch [14]. In this paper, we report that EGCG inhibits both mechanical and biochemical phenotypes of EMT, including cell motility, cell stiffness, and expression of vimentin and Slug in the cells by *in vitro* wound healing assay. Moreover we demonstrate that inhibition of EMT is associated with alteration of cell membrane organization induced by methyl-β-cyclodextrin (MβCD), which depletes cholesterol in cell membranes [15]. The results indicate that the inhibition of EMT with EGCG is a mechanistic step forward prevention of cancer metastasis.

2. Materials and methods

2.1. Cell culture and reagents

A human cancer cell line of non-small cell lung cancer H1299 (large cell carcinoma) was kindly provided by Dr. Naoko Aragane, Saga University, and that of large cell carcinoma Lu99 was obtained from Riken Bioresource Center, Tsukuba, Japan. They were cultured in RPMI1640 medium containing 10% FBS. EGCG was purified from green tea leaves [16]. MβCD was purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-vimentin and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology (CA, USA) and Trevigen (MD, USA), and anti-N-cadherin, and anti-Slug antibodies from Cell Signaling Technology (MA, USA).

2.2. Cell motility as determined by in vitro wound healing assay

H1299 and Lu99 cells were grown in 3.5 cm-culture dish until confluence, and cell migration was determined by *in vitro* wound healing assay, as described previously [17]. The difference between the width at zero and at 24 h was examined by the photos taken under a phase-contrast microscope, ECLIPSE Ti-S (Nikon, Tokyo, Japan), and migration of non-treated cells was expressed as 100%. The values are the means of at least three independent experiments.

2.3. Measurement of cell stiffness

The cells (2×10^5 cells/6 cm-culture plate) were cultured for 2 days, and then treated with EGCG or MβCD in serum free RPMI1640 medium for 4 h. AFM measurement was applied to 25–90 cells, as described previously [7]. Sixteen force-curves per cell were obtained by force-map analysis on the nuclear region of the cell. The spring constant of the cantilever and E value were calculated, as described previously [18]. The Poisson ratio of the cell was taken to be 0.5 [19], and the average values of Young's moduli were obtained from Gaussian fitting for curves.

2.4. Immunocytochemical analysis

After the *in vitro* wound healing assay was conducted, cells were fixed with 4% paraformaldehyde in PBS containing 2% Triton X-100 [20]. Vimentin was visualized by treatment with anti-vimentin antibody as the first antibody, and Alexa-Fluor594-conjugated anti-mouse IgG as the second antibody, and then examined by fluorescent microscope (BIOREVO BZ-9000, Keyence, Osaka, Japan) [20]. Slug was similarly treated with anti-Slug antibody and Alexa-Fluor488-conjugated anti-rabbit IgG.

2.5. Western blot analysis

H1299 and Lu99 cells were treated with EGCG for 24 h. The protein extracts (10 μg) were separated on 12% SDS-gel and blotted onto nitrocellulose membrane (Schleicher & Schuell, Inc., NH, USA) [21]. Vimentin, Slug, N-cadherin, and GAPDH proteins were visualized by the ECL detection system (GE Healthcare, Buckinghamshire, UK) using discrete antibodies.

2.6. Statistics

Statistical analysis for cell stiffness was conducted using non-parametric analysis with Wilcoxon–Mann–Whitney, and the migration was analyzed by a two-sample independent Student's t-test. p-Values lower than 0.01 are considered to be significant.

3. Results

3.1. Inhibition of cell motility in H1299 and Lu99 cells by treatment with EGCG

Cell motility of non-treated H1299 cells was 32.5 ± 6.0 μm for 24 h (Fig. 1A), and expressed as 100% (Fig. 1B). Treatment with 50 and 100 μM EGCG inhibited cell migration from 100% to 67.5% ($p < 0.01$) and 43.7% ($p < 0.01$), respectively. Similarly, treatment of Lu99 cells with 10, 50 and 100 μM EGCG inhibited cell migration from 100% (31.9 ± 6.0 μm) to 81.3% ($p < 0.01$), 71.7% ($p < 0.01$), and 31.5% ($p < 0.01$), respectively. Thus, EGCG dose-dependently inhibited cell motility of two cell lines.

3.2. Increase of cell stiffness in H1299 and Lu99 cells by treatment with EGCG

H1299 and Lu99 cells have highly metastatic potentials. Young's moduli (E: kPa) of non-treated H1299 and Lu99 cells showed a narrow spike peak with average values of 1.24 ± 0.05 and 1.29 ± 0.11 kPa, respectively (Fig. 2). To examine the effects of EGCG on cell stiffness, we used lower concentrations of EGCG than those inducing growth inhibition and morphological change. Treatments of H1299 and Lu99 cells with 5 and 50 μM EGCG for 4 h significantly increased average values of Young's moduli from 1.24 to 2.30 ± 0.07 and 2.25 ± 0.11 kPa in H1299 cells, and from 1.29 to 1.63 ± 0.08 and 2.28 ± 0.09 kPa in Lu99 cells (Fig. 2). The results indicate that EGCG treatment resulted in higher Young's moduli, i.e. a 2-fold increase in cell stiffness in histograms. Thus, we think that EGCG significantly induced rigid elasticity of cell membranes.

3.3. Inhibition of vimentin and Slug expressions in the cells at the leading edge of scratch by treatment with EGCG

The expression of vimentin and Slug in H1299 and Lu99 cells was examined by immunocytochemical staining: The cells at the leading edge of scratch were actively migrating towards the center of the scratch (Fig. 3A and B). Significantly high expression of vimentin and Slug were observed in subpopulation of cells at the leading edge of scratch. Fluorescence intensity of vimentin and Slug in subpopulation of cells increased about 2-fold, compared with that of the other inside cells. Treatment of H1299 and Lu99 cells with 50 μM EGCG significantly inhibited up-regulated expression of vimentin at the edge cells, suggesting the direct effects of EGCG on the cells, and fluorescent intensity in the cells in the other parts was similar to that of the other inside cells (Fig. 3A). Similarly, the expression of Slug was also inhibited by 50 μM EGCG (Fig. 3B). With Lu99 cells, we also found that 50 μM EGCG

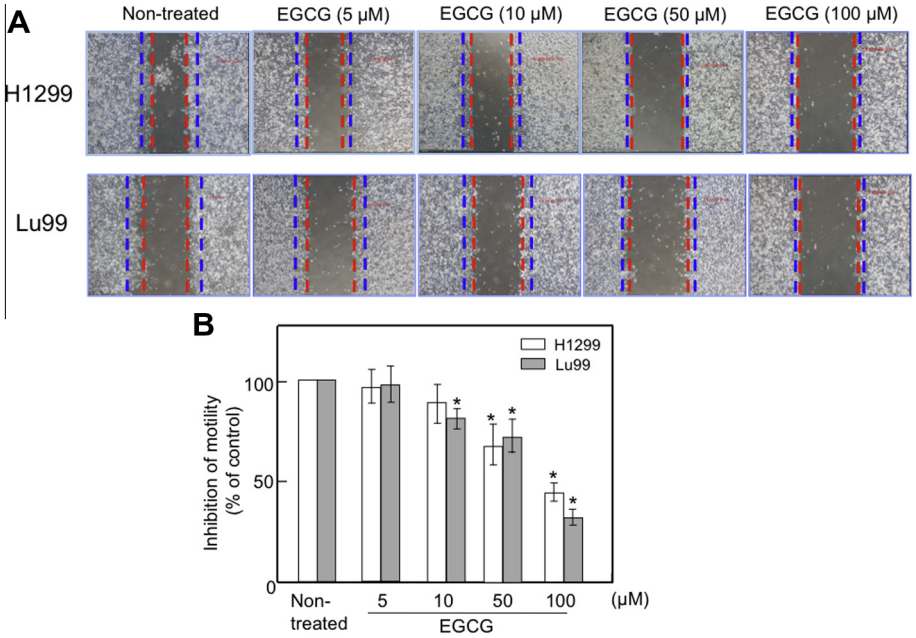

Fig. 1. Inhibition of cell motility in H1299 and Lu99 cells by treatment with EGCG in *in vitro* wound healing assay. (A) Representative wound healing with H1299 cells (upper) and Lu99 cells (lower). Blue dotted line indicates the edge of scratch at 0 h, and red dotted line is the leading edge of scratch 24 h after. (B) Inhibition of cell motility by treatment with EGCG. The values are means of three independent experiments. White bar indicates the result of H1299, and gray bar is that of Lu99. $^{*}p < 0.01$.

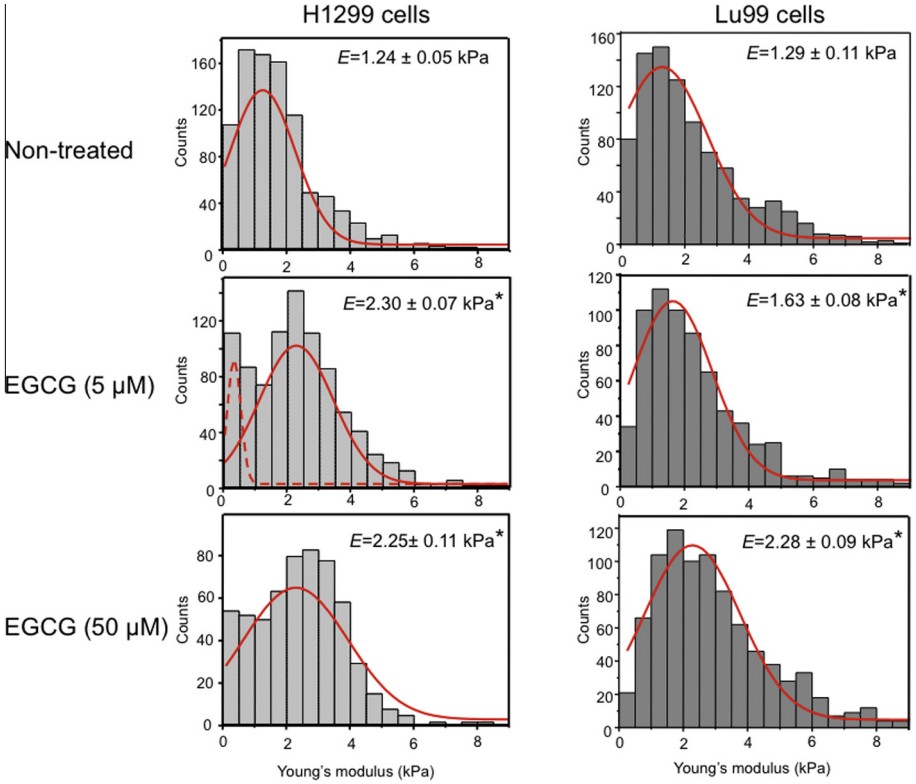

Fig. 2. Increase in Young's moduli of H1299 and Lu99 cells by treatment with EGCG. Young's moduli were obtained from 861 force-curves in 70 non-treated cells, 896 force-curves in 79 cells treated with 5 μM EGCG, and 555 force-curves in 35 cells treated with 50 μM EGCG for H1299 cells (left). Young's moduli were also obtained from 895 force-curves in 62 cells for non-treated cells, 687 force-curves in 57 cells for 5 μM EGCG, and 885 force-curves in 44 cells for 50 μM EGCG for Lu99 cells (right), after treatment for 4 h, as described in Section 2. Red curves are Gaussian fitting curves. $^{*}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly inhibited fluorescent intensity of vimentin and Slug in the cells at the leading edge of the scratch (Supplementary Fig. 1A and B). The results suggest that the metastatic activity of H1299 and Lu99 cells is closely associated with high expression of vimentin and Slug. However, EGCG did not strongly affect the protein levels of vimentin, Slug, or N-cadherin in the cells (Fig. 3C). The

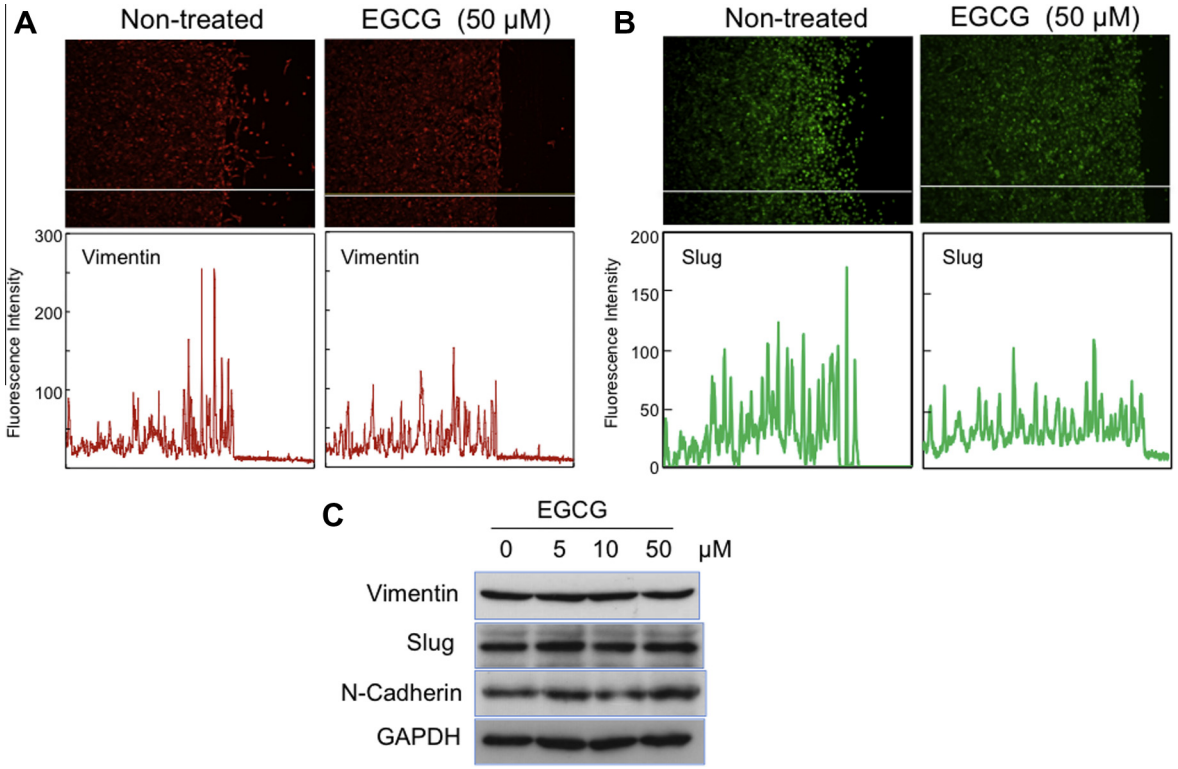

Fig. 3. Inhibition of vimentin and Slug expressions in H1299 cells at the leading edge of scratch after treatment with EGCG. Expression of vimentin (A) and Slug (B) in H1299 cells treated with or without 50 μM EGCG were conducted, as described in Section 2. Lower graph shows fluorescent intensity on the white line of upper photographs. (C) Protein level of vimentin, Slug and N-cadherin in H1299 cells treated with EGCG.

results indicate that EGCG inhibits a critical biomarker that triggers EMT programs.

3.4. Increase in cell stiffness by treatment with MβCD

In light of evidence that EGCG inhibited cell motility and vimentin and Slug expressions in the cells, and increased cell stiffness, we think that cell membrane is biochemically affected by EGCG. We previously reported that EGCG interrupts the interaction of ligands with their receptors (so-called "sealing effects") [22,23], and Weinstein's group recently found that EGCG causes reduction in detergent-insoluble membrane domain, i.e. a decrease in lipid raft in the membrane [24]. In order to study the alteration of cell membrane by EGCG, we used MβCD, which is a reagent disrupting lipid raft in cell membrane as a result of the depletion of cholesterol from the plasma membrane [15]. The effects of MβCD on cell motility, cell stiffness, and expression of vimentin in H1299 cells were studied. Treatment with 5 and 10 mM MβCD significantly inhibited cell migration from 100% (32.5 ± 6.0 μm) to 71.6% and 44.3% (Fig. 4A), and increased Young's moduli from 1.26 ± 0.19 to 2.03 ± 0.39 and 2.81 ± 0.17 kPa (Fig. 4B), respectively. Furthermore, treatment with MβCD inhibited expression of vimentin in subpopulation of cells at the leading edge of scratch (Fig. 4C). The effects of MβCD on cell motility, cell stiffness, and expression of vimentin were also confirmed in Lu99 cells (data not shown). Based on the results, we conclude that EGCG induces alteration of membrane organization, as did MβCD, resulting in rigid elasticity of cell membrane, and that inhibition of cancer metastasis by EGCG is partly based on the inhibition of molecular phenotypes of EMT.

4. Discussion

In this manuscript, we first reported that EGCG inhibits molecular phenotypes of EMT, such as inhibitions of cell motility and expression of vimentin and Slug, and increases in cell stiffness producing rigid elasticity of cell membrane. The inhibition of cell motility with EGCG was found in *in vitro* wound healing assay, but an assay of Transwell cell culture chamber showed the inhibitory effects of EGCG on highly metastatic B16-F10 cells, a mouse melanoma cell variant. For example, treatments with 50, 100 and 200 μM EGCG dose-dependently reduced the cell motility from 100% to 57.1%, 30.3% and 12.6%, respectively [7]. (−)-Epicatechin (EC), an inactive green tea catechin, showed only marginal inhibition, suggesting that the active tea catechins are able to inhibit the motility of various cancer cell lines.

Gimzewski's group first studied cell stiffness using AFM of mesothelial (normal) and metastatic cells taken directly from patient's body samples, including one pancreatic adenocarcinoma, one breast adenocarcinoma, three lung adenocarcinomas, and four ovarian adenocarcinomas. Although the average Young's moduli for normal and metastatic cells were 2.53 ± 1.23 and 0.41 ± 0.18 kPa, respectively, treatment of these various cells with green tea catechins showed the average Young's moduli for normal and metastatic cells were 2.48 ± 1.37 and 2.54 ± 1.47 kPa, respectively [4], indicating that EGCG causes a significant increase in Young's moduli of metastatic cells, whereas EGCG did not show any increase in Young's moduli of normal cells. Since the effects of EGCG on metastatic activity are found in various human cancer cell lines, EGCG possesses a very selective action on cancer cells, probably inducing rigid elasticity in cancer cell membrane [4]. Recently, the cell stiffness of cancer cells can be studied using nanobiophysical techniques, such as AFM, a magnetic tweezer system and micropipette aspiration [5–7,11]. Various investigators also studied the relationship between cell stiffness and cancer metastasis: the expression of metastatic suppressor genes, such as *type III tumor growth factor-β (TGF-β) receptor* increased cell stiffness of ovarian cancer cell lines [11], and *breast cancer metastatic suppressor 1* significantly suppressed invasiveness and metastasis of human breast cancer cell line [25].

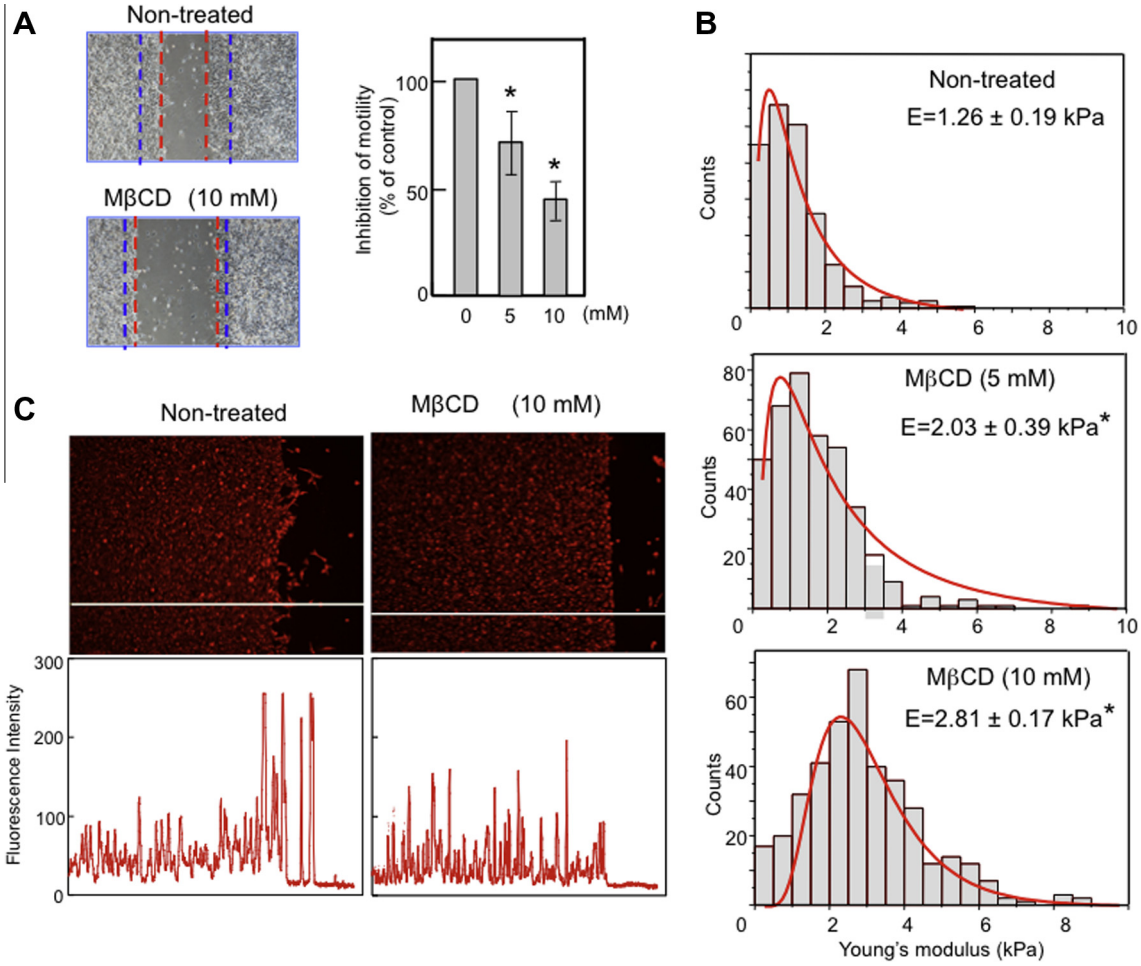

Fig. 4. Increase in cell stiffness by treatment with MβCD. (A) Inhibition of cell migration with MβCD. Blue dotted and red dotted lines are the same as Fig. 1 in the photographs (left), and the graph shows the result of cell migration (right), $^{*}p < 0.0001$. (B) Histogram of Young's moduli obtained from 861 force-curves in 70 non-treated H1299 cells, 392 force-curves in 26 cells treated with 5 mM MβCD, and 382 force-curves in 35 cells treated with 10 mM MβCD. Red curves are log-normal fitting curves. (C) Inhibition of vimentin expression in H1299 cells treated with 5 mM MβCD. The graph shows fluorescent intensity on the white line, as described in Fig. 3.

Vimentin, Slug, and N-cadherin are mesenchymal proteins belonging to the molecular phenotypes of EMT [9,12]. The expression of vimentin and Slug in human non-small lung cancer cell lines H1299 and Lu99 was associated with high migration potential and lower Young's modulus, and the effects were inhibited with EGCG and other green tea catechins, maintaining the steady-state level of mesenchymal proteins. Although Gimzewski's group reported that green tea extract increased cell stiffness and induced reorganization of actin filaments in human lung cancer cells [4], we did not find any significant difference in actin filaments between non-treated H1299 and EGCG-treated H1299, as determined by staining with AF488-phalloidin 4 h after treatment (data not shown).

How cell stiffness and rigid elasticity are induced in the cells and how those effects are induced with green tea catechins are important biochemical subjects. Based on our hypothesis on the sealing effects of EGCG, it is worthwhile to study the effects of the lipid raft with MβCD, which induces alteration of membrane organization and disintegration of lipid raft by depletion of cholesterol from the plasma membrane. In light of evidence that MβCD suppresses migration of U-251 MG glioma cells by stimulation of CD44 shedding [26], our results strongly suggest that membrane organization directly reflects cell motility and cell stiffness. This is also confirmed by a recent report showing that high-resolution matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI/IMS) revealed the different composition of phosphatidylinositol in invasive regions [27]. Metastasis is the primary cause of death in cases of human cancer and lung cancer is the leading cause of death in the world. We showed in this paper that nanobiophysical techniques such as AFM contribute to biochemical understanding of cancer metastasis as one of the EMT phenotypes. Finally, we conclude that the inhibition of mechanical and biochemical EMT phenotypes with EGCG is a mechanism-based inhibition of cancer metastasis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.094.

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