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(−)-Epigallocatechin-3-Gallate, a Polyphenolic Compound From Green Tea, Inhibits Fibroblast Adhesion and Migration Through Multiple Mechanisms

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Abstract It is increasingly evident that the stromal cells are involved in key metastatic processes of melanoma and some malignant solid tumors. (−)-Epigallocatechin-3-gallate (EGCG), a polyphenolic compound from green tea, has been shown to have anti-tumor activity, inhibiting adhesion, migration, and proliferation of tumor cells. However, little attention has been paid on its effects on stromal cells. In the present study, we determined the effects of EGCG on stromal fibroblasts. We showed that fibroblast adhesion to collagen, fibronectin, and fibrinogen were inhibited by EGCG. One of the possible mechanisms is binding of EGCG to fibronectin and fibrinogen but not to collagen. We then focused how EGCG affected fibroblast adhesion to collagen. EGCG treatment attenuated the antibody binding to fibroblast's integrin $\alpha2\beta1$, indicating EGCG may affect the expression and affinity of integrin $\alpha2\beta1$. Moreover, intracellular H_2O_2 level was decreased by EGCG treatment, suggesting that the tonic maintenance of intracellular H_2O_2 may be required for cell adhesion to collagen. In parallel, collagen-induced FAK phosphorylation, actin cytoskeleton reorganization in fibroblasts, migration and matrix metalloproteinase(s) (MMPs) activity were also affected by EGCG. Tubular networks formed by melanoma cells grown on three-dimensional Matrigel were also disrupted when fibroblasts were treated with EGCG in a non-contact coculture system. Taken together, we provided here the first evidence that EGCG is an effective inhibitor on behaviors of the stromal fibroblasts, affecting their adhesion and migration. The inhibitory activity of EGCG may contribute to its anti-tumor activity. The findings and concepts disclosed here may provide important basis for a further experiment towards understanding tumor–stroma interaction. J. Cell. Biochem. 96: 183–197, 2005.

Key words: adhesion; (−)-epigallocatechin-3-gallate; EGCG; fibroblast; integrin; matrix; melanoma

Cutaneous melanomas and some malignant solid tumors are notorious for their tendency to metastasis. A malignant solid tumor is generally a compact mass, containing a complex mixture of neoplastic cells and tumor stroma [Ruiter et al., 2002]. The tumor stroma consists of microvessels, fibroblast (-like) cells, inflammatory cells, and extracellular matrices (ECMs). Although past research has revealed that growth factors, adhesion molecules, proteases, and other related components play an important role in the pathogenesis of melanoma, it is increasingly evident that the stromal cells are also involved in key metastatic processes, including proliferation, matrix degradation, and migration [Liotta et al., 1991; Fidler, 1995].

Fibroblasts organize tumor stroma formation by production of extracellular matrix components and are responsible for many of its functions [Ruiter et al., 2002]. In melanoma development and progression, the interactions between melanoma cells and fibroblasts are through several pathways. Fibroblasts serve as

Abbreviations used: DHR 123, dihydrorhodamine 123; ECG, (−)-epicatechin-3-gallate; ECM, extracellular matrix; EGCG, (−)-epigallocatechin-3-gallate; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; H_2O_2, hydrogen peroxide; mAb, monoclonal antibody; MMP(s), matrix metalloproteinase(s); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NIgG, nonimmune IgG; PAF, paraformaldehyde.

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a "bad neighbor" of melanocyte and a positive stimulator for cancer cells to proliferate, survive, and migrate [Hazan et al., 2000; Li and Herlyn, 2000]. Tumor growth can not be sustained unless the tumor cells attract and stimulate fibroblasts. For example, fibroblasts are a rich source of growth factors. When stimulated, fibroblasts can produce a pool of growth factors (insulin-like growth factor-1, hepatocyte growth factor, basic fibroblast growth factor, and eodothelin-3), which may affect tumor progression [Halaban, 1996]. In addition, activated fibroblasts can proliferate, produce ECM and are converted into myofibroblast [Folberg et al., 1992].

Green tea contains many polyphenols such as flavanols (catechins), flavonols, flavandiols, and phenolic acid. These polyphenols account for a third of the dry weight of the leaves. The major green tea catechins are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (+)-catechin [Graham, 1992]. These compounds have shown cytostatic properties in several tumor models. One facet of the antitumor activities exerted by EGCG seems to be attributed to blocking of the EGF receptor [Sachinidis et al., 2000]. A recent study has demonstrated that EGCG inhibits focal adhesion kinase activity, indicating that it interferes with cancer cell adhesion and movement processes [Liu et al., 2001; Suzuki and Isemura, 2001]. Moreover, EGCG has been described to inhibit matrix metalloproteinase (MMP)-2 and -9, resulting in a significant reduction of the invasive behavior of gelatinase-expression cancer cells [Benelli et al., 2002]. Many studies have been focused on the effects of EGCG on tumor cells, however little attention has been paid to the effect of EGCG on stromal cells such as fibroblasts.

As described earlier, the tumor microenvironment affects tumor development. Ruiter et al. [2002] have proposed that fibroblasts are involved in the melanoma growth and progression in several stages. These include recruitment of resident fibroblasts and activation and proliferation of recruited fibroblasts, etc. Moreover, fibroblasts and keratinocytes were shown to contribute both to the synthesis and the degradation of the molecules important for the integrity of this skin site [Bosset et al., 2003]. Therefore, in the present study we examined the effects of EGCG on skin fibroblasts. We found that fibroblast adhesion to matrix proteins such as collagen, fibronectin, and fibrinogen were inhibited by EGCG. The possible action mechanisms of EGCG on fibroblast adhesion were elucidated. The integrin $\alpha2\beta1$, a receptor expressed on fibroblasts that binds to collagen, was inhibited by EGCG. Moreover, focal adhesion kinase (FAK) phosphorylation and actin cytoskeleton organization were affected. Since several polyphenols in green tea have been shown to have antioxidant activities [Katiyar and Elmets, 2001; Katiyar et al., 2001], we also examined whether EGCG affected intracellular hydrogen peroxide (H_2O_2) generation. Finally, we determined whether EGCG affected fibroblast migration and melanoma cell behavior.

EXPERIMENTAL PROCEDURES

Materials

EGCG, bovine serum albumin (BSA), bovine type I collagen, human plasma fibrinogen, FITC-phalloidin, aprotinin, leupeptin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF), sodium fluoride (NaF), and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO). Human plasma fibronectin was from Invitrogen Life Technologies (Carlsbad, CA). Antibodies raised against phospho-ERK1/2 (E-4) and FAK were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody raised against phospho-FAK (Tyr397) was from Upstate Biotech, Inc. (Lake Placid, NY). Antibody raised against ERK1/2 was from R&D systems, Inc. (Minneapolis, MN). Antibodies raised against integrin $\alpha2\beta1$, nonimmune IgG (NIgG), FITC-conjugated goat anti-human secondary antibody were purchased from Chemicon (Temecula, CA). 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF/AM), dihydrorhodamine 123 (DHR 123), and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) were from Molecular Probes (Eugene, OR). Anti-MMP-1 and MMP-2 antibodies were purchased from Calbiochem (EMD Bioscience, Inc., San Diego, CA).

Cell Culture and Preparation of Suspended Cells

Human foreskin fibroblast cell line (Hs68) and human metastatic melanoma cell line (A2058) were purchased from Food Industry

Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (GibcoBRL, Invitrogen Life Technologies, Carlsbad, CA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrish, Sigma Chemical Co., St. Louis, MO). For most experiments, cells reaching a 90%–95% of confluency were starved in DMEM at 37°C for 24 h. Cells were detached by trypsin/EDTA and collected by centrifugation. In some experiments, collected cells were maintained in suspension at 37°C for 30 min for further analysis.

Adhesion Assay

Ninety-six-well plates (Costar, Cambridge, MA) were coated with 50 μl type I collagen (collagen, 20 μg/ml), fibronectin (20 μg/ml), fibrinogen (20 μg/ml), or 1% BSA in PBS at 4°C for overnight. After a brief wash with PBS, the plates were blocked with 10% BSA at 37°C for 1 h. Suspended fibroblasts were labeled with BCECF/AM (10 μg/ml) for 30 min at 37°C. The labeled cells were washed and resuspended in DMEM to a density of 1.5×10^5 cells/ml. The suspended cells (95 μl) were incubated with 5 μl PBS or various concentrations of EGCG, ECG, and (+)-catechin for 30 min at 37°C. Control or the pretreated fibroblasts were applied onto the collagen-, fibronectin-, or fibrinogen-precoated 96-well plates and incubated for 2 h at 37°C to allow adhesion in the absence or presence of the indicated catechins. After washing twice with PBS, the nonadherent cells were removed by aspiration and the 96-well plates were subjected to measurement by Wallac Victor 3 1420 multilabel counter (Perkin Elmer, Turku, Finland) using excitation and emission wavelength at 485 and 535 nm, respectively. For some experiments, cells were pretreated with EGCG for 30 min and followed by centrifugation to remove EGCG. Cells were loaded with BCECF/AM and allowed to adhesion in the absence of EGCG. Or, matrix protein-coated plate was blocked with 10% BSA at 37°C for 1.5 h. After a brief wash with PBS, the plates were incubated with EGCG for an additional 2 h and followed by a wash with PBS to remove unbound EGCG. Then, BCECF/AM-loaded cells were allowed to adhesion and measured as described above.

LDH Release and Cell Viability Assays

LDH release in fibroblast's culture media was determined by Promega cytotoxicity assay kit (Promega Corporation, WI) according to the protocol provided by manufacturer. Cell viability was assayed as previously described [Wu et al., 2001]. Briefly, the cells were incubated with 0.5 mg/ml MTT for 2 h at 37°C. Formazan crystals resulting from MTT reduction were dissolved by adding DMSO. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 550 nm.

Morphological Analysis of Fibroblasts

Twenty-four well culture plate (Costar) were coated with collagen, fibronectin, and fibrinogen and blocked with 10% BSA as described above. Fibroblasts pretreated with PBS or EGCG for 1 h were seeded and allowed to adhesion on plate for an additional 1.5 h. After that, medium was removed and cells were fixed with 1% paraformaldyhe (PAF) for 30 min at room temperature. After a wash with PBS, cells were observed under a Leica DMIL® phase-contrast microscope and photographed by a digital camera.

Flow Cytometric Analysis of Antibodies Binding to Integrin

Fibroblasts in monolayer culture were washed with PBS, trypsinized and collected by centrifugation. The suspended cells were treated with or without EGCG and (+)-catechin at 37°C for 1 h. Then, cells were centrifuged, resuspended in 1% BSA-containing PBS, and fixed with 1% PAF for 30 min at 4°C. After a wash, cells were labeled with primary α2β1 integrin mAbs (1:50, final 20 μg/ml) or control nonimmune IgG (as a negative control) for 1 h at 4°C. Labeled cells were washed with 1% BSA-containing PBS and then incubated with secondary FITC-conjugated goat anti-mouse IgG at room temperature for 1.5 h with a continuous shaking. After incubation, cells were washed, centrifuged, resuspended in PBS, and analyzed immediately by a Partec CyFlow ML cytometer (Partech GmBH, Munster, Germany) using excitation and emission wavelength at 488 and 525 nm, respectively. Fluorescence signals from 10,000 cells were collected to calculate mean fluorescence intensity of a single cell.

Flow Cytometric Analysis of Intracellular H_2O_2 Level

Intracellular production of H_2O_2 was assayed as previously described [Katiyar et al., 2001] with minor modification. Briefly, resuspended

fibroblasts loaded with DHR 123 (10 μg/ml) or CM-H_2DCFDA (10 μg/ml) were incubated at 37°C for 30 min. After centrifugation, cells were resuspended in a tube or allowed to adhere at 37°C for 1 h in the presence or absence of EGCG on a 3.5 cm dish, which was coated with PBS or collagen. After that, cells were collected by scraping and centrifugation. The cell pellets were resuspended in 1 ml PBS and then analyzed immediately by the Partec CyFlow ML cytometer (Partech GmBH) at excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence signals of 10,000 cells were collected to calculate mean fluorescence intensity of a single cell.

Cell Lysate Preparation and Western Blot Analysis

Fibroblasts suspended in medium or adhered to collagen-precoated petri dishes (3.5 cm, Falcon) in the presence or absence of EGCG were washed with prechilled PBS and lysed in radioimmunoprecipitation assay buffer [20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1 mM sodium fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μg/ml aprotinin and leupeptin (freshly prepared)]. After sonication, the lysate was centrifuged (14,000*g* for 15 min at 4°C), and supernatant was transferred to a tube. The protein content was quantified by Pierce protein assay kit (Pierce, Rockford, IL). Total protein was separated by electrophoresis on SDS–10% polyacrylamide gels and the proteins were electroblotted onto PVDF membranes and then probed using primary anti-phospho-ERK (0.2 μg/ml) or anti-phospho-FAK (1 μg/ml) mAbs. Immunoblots were detected by enhanced chemiluminescence (Chemiluminescence Reagent Plus from NEN, Boston, MA). For detection of total FAK and ERK1/2, the PVDF membrane was stripped at 60°C for 30 min with a striping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol). Membranes were washed with TBS-T (Tris-buffered saline/0.05% tween 20) for three times and probed with anti-FAK (1 μg/ml) and anti-ERK1/2 (0.5 μg/ml) antibodies and developed as described above.

Immunofluorescence Microscopy

For staining actin cytoskeleton, the procedure has been described previously in detail [Lampugnani et al., 1995]. Briefly, trypsinized fibroblasts were suspended at 37°C for 30 min, and then pretreated with or without EGCG for an additional 1 h. Cells were then allowed to adhere on glass coverslips precoated with collagen (20 μg/ml) for 1 h. Cells were then washed, fixed with 1% PAF for 20 min, and permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 3% BSA, cells were incubated with FITC-conjugated phalloidin (Sigma, 1:200). Coverslips were mounted and the immunofluorescence images were analyzed under an Olympus BX51 microscope and photographed using a Spot RT Camera System (Diagnostics Instruments, Inc., Sterling Height, MI).

Migration Assays

Migration assays with fibroblasts were performed by scratch assay and using a modified Boyden chamber model (Transwell apparatus, 8.0 μm pore size, Costar) [Leavesley et al., 1993]. Scratch assay was performed in 24-well plates as previously described [Cha et al., 1996] with minor modification. The cells were grown to confluence and a 2-mm broad denuded scratch was produced in the culture with a pipette tip. After washing with PBS and serum-free medium to remove all floating cells, the cells at the cut edge were allowed to migrate into the empty zone for 24 h. Photographs of identical locations within each scratch were taken under a phase-contrast microscope. For detection of fibroblast migration in the Tranwell, the lower face of polycarbonate filters (Transwell insert) were coated with type I collagen (0.4 μg) or fibronectin (0.4 μg) for 30 min in the laminar flow hood. The lower chamber was filled with 0.6 ml of serum-free medium. Fibroblasts (5×10^4 cells, 200 μl) were plated to the upper chamber. After 20 h of incubation, all nonmigrant cells were removed from the upper face of the Traswell membrane with a cotton swab and migrant cells were fixed and stained with 0.5% toluidene blue in 4% PAF. Migration was quantified by counting the number of stained cells per ×100 field (high power field, HPF) with a phase-contrast microscope (Leica DMIL®) and photographed.

Gelatin Zymography

SDS-substrate zymography electrophoresis was performed using a previously described method [Yan et al., 2000]. Briefly, samples of medium conditioned by cell culture under different experimental conditions were

centrifuged. Sample in equal volume were separated on an 8.5% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 (in 50 mM Tris-HCl) for 30 min. Substrate digestion was performed by incubating the gel in 50 mM Tris-HCl, pH 7.6, containing 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 1% Triton X-100 and 0.02% NaN_3 at 37°C for 24 h. The gel was stained with 0.1% Coomassie Brilliant Blue R250 (Sigma Co.), and the location of gelatinolytic activity was detected as clear bands. Arbitrary density of an individual cleavage band was determined by scanning densitometry using ImageQuant software (General Electronic Co., Fairfield, CT).

Melanoma Cell Tube Formation in Non-Contact Cocultures

Non-contact cocultures were performed using a Transwell model (Costar), which allowed bidirectional diffusion of soluble factors. Fibroblasts were first cultured in the lower chamber, washed and incubated with serum-free DMEM when reaching to 90% of confluency. In the meantime, the upper chamber (polycarbonate filters) were coated with Matrigel (BD Biosciences, Bedford, MA), which was diluted to 4 mg/ml with serum-free DMEM at 4°C. An aliquote (30 μl) of Matrigel was added to each filter insert and incubated at 37°C for 30 min to form a uniform three-dimensional gel. Melanoma cells (5×10^4 in 200 μl DMEM) were plated in the upper chamber and assembled for cocultivation for 20 h. The viability of fibroblasts in the lower chamber was monitored by MTT assay. Tubular networks formed by melanoma cells in the upper chamber were washed, fixed with 4% PAF and then photographed under a microscope. The degree of tube formation (number of junctions) was quantified by counting the number of junctions per ×100 field under a phase-contrast microscope.

RESULTS

EGCG and ECG but not (+)-Catechin Inhibited Fibroblast Adhesion to Matrix Proteins

A growing body of evidence has suggested that activated fibroblasts proliferate and migrate to premalignant tissue peripheries where they synthesize new matrix components (e.g., type I collagen and fibronectin) in response to a variety of growth factors [Martin, 1997; Coussens and Werb, 2002]. We therefore investigated whether polyphenolic compounds such as EGCG, ECG, and (+)-catechin will affect fibroblast adhesion to various matrix proteins. Figure 1A shows that fibroblast adhered to type I collagen (collagen), fibronectin, and fibrinogen as determined by cell adhesion assay. EGCG and ECG (50 μM) significantly inhibited fibroblast adhesion to collagen, fibronectin, and fibrinogen. However, (+)-catechin, an abundant polyphenolic component in fruits such as apple and grape, showed no inhibitory effect on fibroblast adhesion to these matrix proteins. The inhibitory effect of EGCG on cell adhesion to these matrix proteins was in a concentration-dependent manner (Fig. 1B). It seems that EGCG produced more pronounced inhibitory effects on cell adhesion to fibronectin and fibrinogen.

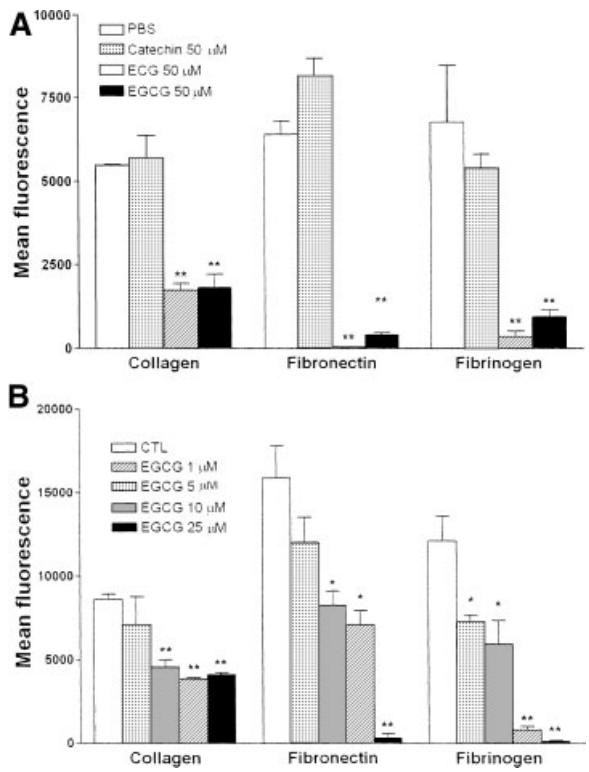

Fig. 1. (−)-Epigallocatechin-3-gallate (EGCG) inhibited fibroblast adhesion to matrix proteins. **A**: Effects of polyphenolic compounds on fibroblast adhesion. Suspended fibroblasts were loaded with BCECF/AM and pretreated with EGCG, ECG, and (+)-catechin. Cell adhesion was performed by adding of cells to 96-well plates precoated with collagen, fibronectin, or fibrinogen (20 μg/ml) for 2 h at 37°C and measured by a fluorescence plate reader. **B**: EGCG dose-dependently inhibited fibroblast adhesion. Cells treated with EGCG were allowed to adhesion and measured as described in (A). Results were expressed as fluorescence intensity and were mean ± SEM (n = 5). $^*P < 0.05$ and $^{**}P < 0.01$ versus PBS control.

It has been reported that EGCG at 1–10 μM could trigger pro-apoptotic events in certain types of cells [Sartor et al., 2004]. Therefore, to exclude the possibility that EGCG has cytotoxic or pro-apoptotic effects on fibroblasts, we performed cytotoxicity and MTT assays. As shown in Figure 2A, EGCG treatment did notÂincrease lactate dehydrogenase (LDH) release in culture medium, but rather, it decreased LDH release as compared with control (panel a), suggesting that EGCG is not toxic and may have protective effect on skin fibroblasts. The MTT assay also demonstrated that the viability of fibroblasts cultured in serum-free or serum-containing medium was not affected when incubated with EGCG (panel b and data not shown). The results excluded the possibility that the inhibitory effects of EGCG were due to its cytotoxic or pro-apoptotic activities.

To further elucidate the possible mechanisms of EGCG in inhibiting fibroblast adhesion to different matrix proteins, fibroblasts were analyzed under a phase-contrast microscope. Figure 2B shows that fibroblasts fully spread when adhered to collagen, fibronectin, and fibrinogen (panels a–c, PBS controls). However, some cells on plates precoated with collagen rounded up and showed some protrusion at cell periphery in the presence of EGCG (25 and 50 μM) (panel a). On the other hand, cell adhesion to fibronectin and fibrinogen were obviously inhibited in the presence of EGCG. Most cells rounded up in the presence of EGCG (panels b and c).

EGCG Interfered With Fibroblast–ECM Interactions Through Multiple Mechanisms

Sazuka et al. [1998] have demonstrated that EGCG impairs adhesion and/or spreading of

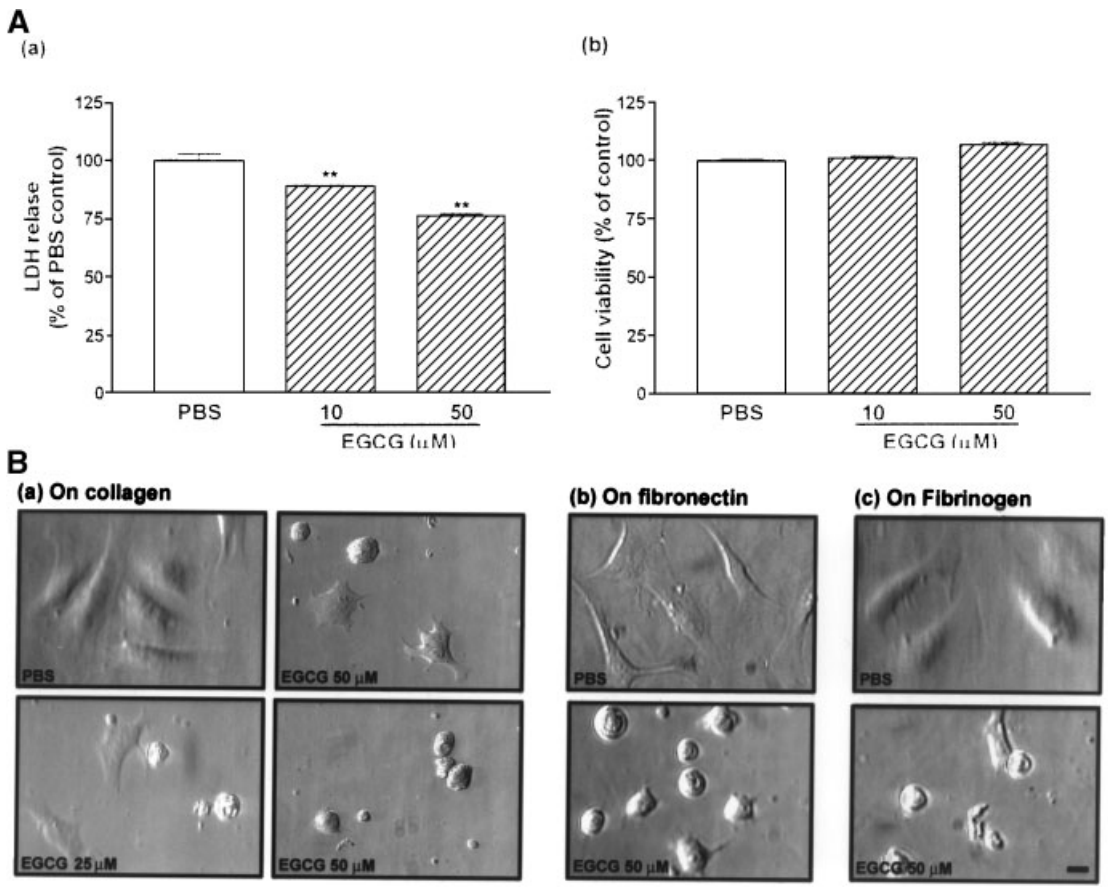

Fig. 2. Effect of EGCG on cell viability and morphology. **A**: Fibroblasts were treated with EGCG for 24 h. Culture media were then removed for (**a**) cytotoxicity assay and the remaining adherent cells were determined by (**b**) MTT assay, as described in ''Experimental Procedures.'' Results were expressed as the percentage of control and were mean ± SEM (n = 3). $^{**}P < 0.01$ versus PBS control. **B**: Morphological analysis of fibroblast adhesion. Cells treated with PBS or EGCG for 1 h were seeded and allowed to adhesion on (**a**) collagen-, (**b**) fibronectin- or (**c**) fibrinogen-precoated plates at 37°C for an additional 1.5 h in the absence or presence of EGCG. Cells were then fixed and photographed under a phase-contrast microscope. Bar, 50 μm.

mouse lung carcinoma and melanoma cells through the interaction with the carboxyl-terminal domain of fibronectin. Suzuki and Isemura [2001] also demonstrated that EGCG inhibited melanoma cell adhesion through binding to laminin. In Figure 2B, we showed that fibroblasts rounded up in the presence of EGCG. We therefore determined if EGCG affects cell surface receptor expression/affinity or binds to ECM proteins. Fibroblasts were pretreated with EGCG and followed by centrifugation to remove unbound EGCG. Cells were allowed to adhere to the indicated ECM proteins in the absence of EGCG. As shown in Figure 3A, EGCG still inhibited cell adhesion to collagen, fibronectin, and fibrinogen. On the other hand,

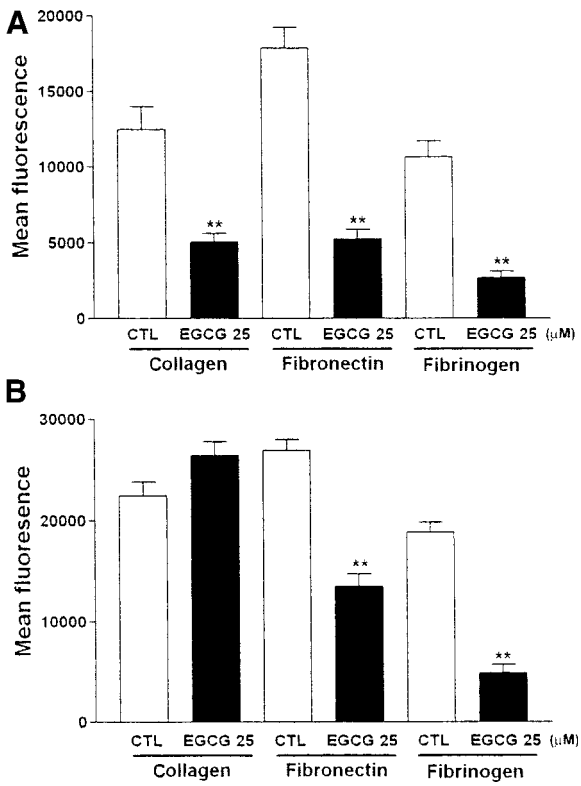

Fig. 3. Differential inhibitory effects of EGCG on fibroblast adhesion to matrix proteins. **A**: Cells were pretreated with PBS or EGCG (25 μM) for 30 min and followed by centrifugation to remove unbound EGCG. Cells were then allowed to adhere to collagen-, fibronectin-, and fibrinogen-precoated plates. After 2 h of adhesion, number of adherent cells was measured as described in "Experimental Procedures." **B**: Collagen-, fibronectin-, and fibrinogen-precoated plates were incubated with PBS or EGCG (25 μM) at 37°C for 1.5 h. After a wash to remove unbound EGCG, fibroblast adhesion to the indicated matrix proteins was performed. Number of adherent cells was measured using a fluorescence plate reader. All Data shown in (A) and (B) were expressed as fluorescence intensity and were mean ± SEM (n = 3). $^{**}P < 0.01$ as compared with controls.

when ECM protein-precoated plates were incubated with EGCG at the beginning and followed by a brief wash to remove unbound EGCG, only cell adhesion to fibronectin and fibrinogen were affected (Fig. 3B). The results indicate that EGCG may impair fibroblast interaction with ECM proteins via affecting cell surface receptor expression/affinity or binding to fibronectin and fibrinogen, but not to collagen.

EGCG Inhibited Antibodies Binding to α2β1 Integrin Receptor

Two distinct receptors have been implicated in mediating collagen binding to cells. These include α2β1 and α1β1 integrin receptors [Plow et al., 2000]. Since EGCG apparently did not bind to collagen but showed inhibiting the adhesion of EGCG-pretreated fibroblasts to collagen (Fig. 3), we next examined whether EGCG affected fibroblast collagen receptor. Flow cytometric analysis showed that binding of anti-integrin α2β1 mAb, i.e., the mean fluorescence intensity, significantly increased as compared with nonimmune IgG (NIgG)-binding control cells (Fig. 4A, a), suggesting the presence of α2β1 integrin on fibroblasts. However, the fluorescence intensity was attenuated upon EGCG treatment (Fig. 4A, b), indicating EGCG compromises mAb binding to integrin α2β1 receptor on fibroblasts. On the contrary, (+)-catechin did not show any inhibition on mAb binding to integrin α2β1 expressed on fibroblasts (Fig. 4A, c). Quantitative analysis showed that the decrease of mean fluorescence was about 10%–15% of PBS control in the presence of EGCG (Fig. 4B).

EGCG Treatment Caused the Decrease of Intracellular H_2O_2 Level

Recently, "redox" signaling has come into focus in cellular biology studies. Chiarugi et al. [2003] have shown that reactive oxygen species are as essential mediators of cell adhesion. The authors demonstrated that intracellular H_2O_2 level increases when NIH-3T3 fibroblast adhesion to fibronectin via integrin α5β1 receptor. In our experiments, we measured intracellular H_2O_2 level during cell adhesion by using a specific dye, namely dihydrorhodamine 123 (DHR 123), and followed determined by flow cytometry. However, we did not detect significant intracellular H_2O_2 generation while fibroblast adhered to collagen. Surprisingly, intracellular H_2O_2 level was decreased in

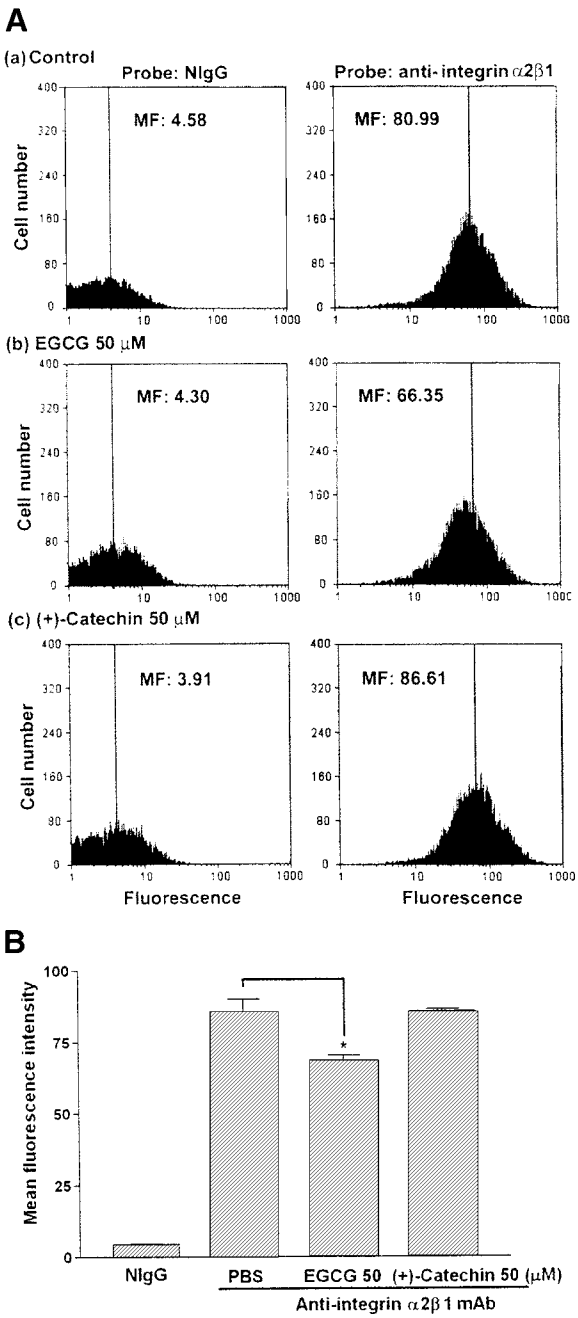

Fig. 4. EGCG inhibited integrin α2β1 expression. **A**: Flow cytometric analysis of integrin α2β1 expression. Cells were treated with PBS, EGCG, or (+)-catechin (50 μM for each) for 1 h. After incubation, cells were then fixed and incubated with nonimmune IgG (NIgG) or anti-integrin α2β1 mAb at 4°C for 1 h, followed by FITC-conjugated Abs and analyzed immediately by flow cytometry. Each histogram is a representative of three to four separate experiments. MF: mean fluorescence intensity of cells. **B**: Quantitative analysis of the binding of NIgG and anti-integrin α2β1 mAb to fibroblasts in the presence of PBS, EGCG, or (+)-catechin. Data were presented as mean fluorescence intensity ±SEM (n = 3). $^*P < 0.05$ as compared with controls.

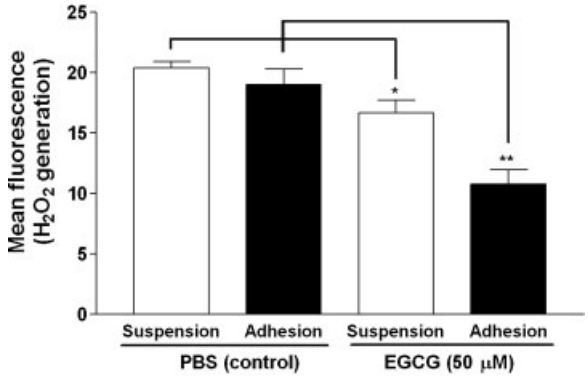

Fig. 5. Effect of EGCG on intracellular H_2O_2 level in fibroblasts. Trypsinized cells were either suspended in DMEM or allowed to adhesion on collagen-precoated dishes in the presence or absence of EGCG (50 μM) at 37°C for 1 h. After loading with DHR 123, cells were washed, centrifuged, resuspended in PBS and analyzed immediately by flow cytometry. Data were presented as mean fluorescence intensity and were mean ± SEM SEM (n = 3), $^*P < 0.05$, $^{**}P < 0.01$ as compared with controls.

suspended and adherent cells upon EGCG treatment (Fig. 5). Although the increase of intracellular H_2O_2 of the adherent cells was not detected in our experimental system, the results suggest that the maintenance of intracellular H_2O_2 level may be required in fibroblast adhesion to collagen.

EGCG Inhibited FAK Phosphorylation and Actin Cytoskeleton Organization During Cell Adhesion

FAK plays a prominent role in integrin signaling. An increase in major phosphorylation site (Tyr^{397}) as well as other sites within FAK is observed after ECM ligation of integrins at the cell surface [Parsons, 2003]. It has also been reported that integrin and components of focal adhesion complex associate with actin cytoskeleton, which transmits biological signals and mechanical forces across the plasma membrane [Aplin et al., 1998; Calderwood et al., 2000]. Since the binding of antibodies to fibroblast integrin α2β1 receptor was blocked by EGCG (Fig. 4), we therefore attempted to determine whether FAK phosphorylation was affected by EGCG. As shown in Figure 6A, the phosphorylation of FAK at Tyr^{397} was not obvious in suspended cells, but was increased while cells adhered to collagen in a time-dependent manner. The increase of FAK phosphorylation in cells was inhibited in the presence of EGCG, whereas the total FAK was unchanged. Quantitative analysis showed that

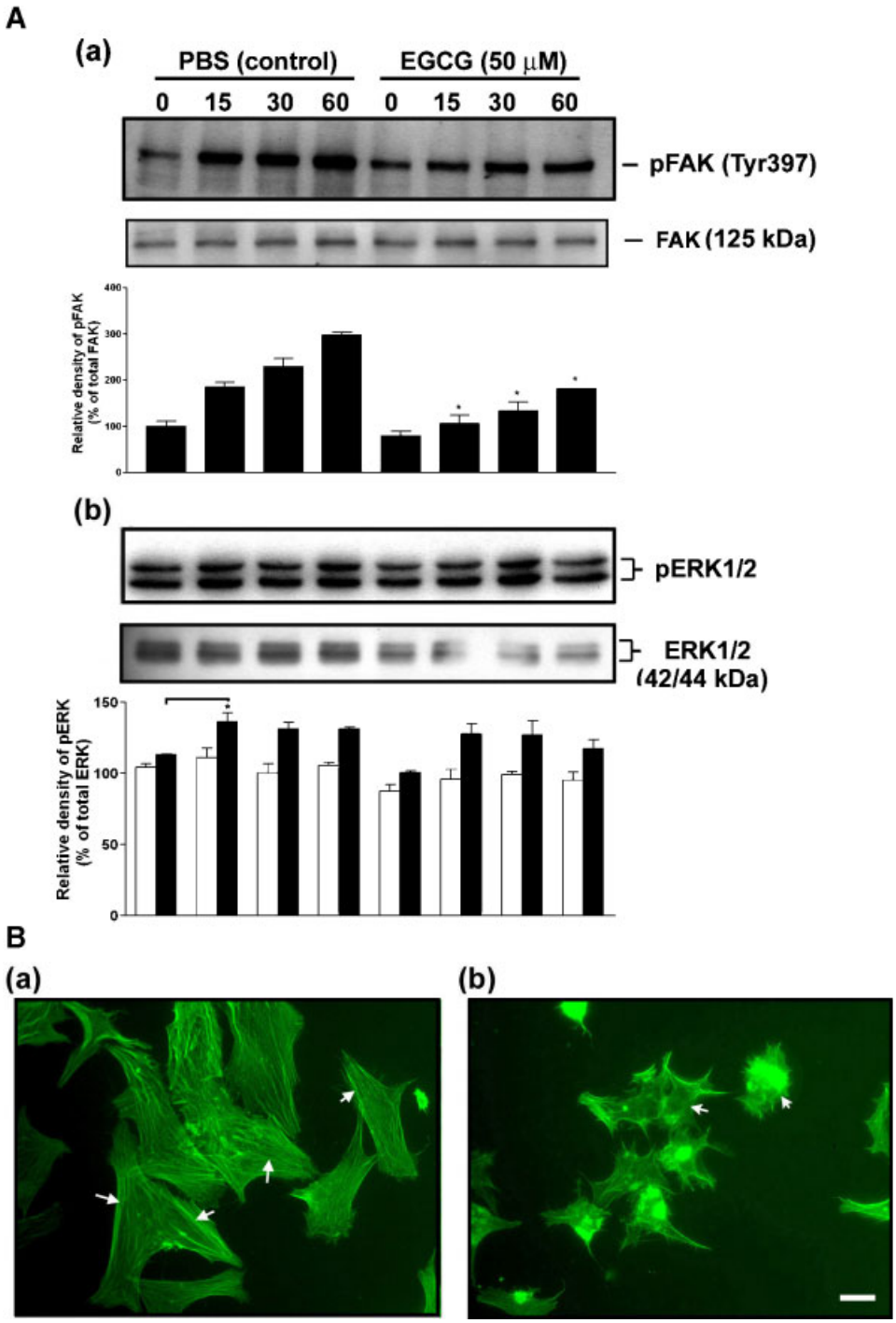

Fig. 6. Effects of EGCG on phosphorylation of FAK and ERK1/2 and actin cytoskeleton reorganization. **A**: Fibroblasts suspended in medium or adhered to collagen-precoated dishes in the presence or absence of EGCG were collected and subjected to analysis by SDS–10% polyacrylamide gel. After blotting to membrane, immunoblotting was performed using mAbs (**a**) anti-phospho-FAK (Tyr^{397}) and anti-total FAK, (**b**) anti-phospho-ERK1/2 and anti-total ERK1/2. Each lower panel in (a) and (b) was the quantitative analysis of phosphorylation by densitometry and was expressed as the percentage of each total protein. Each blot is the representative of three experiments. $^*P < 0.05$ as compared with controls. **B**: Suspended fibroblasts pretreated with PBS (control) or EGCG (50 μM) for 1 h were seeded and allowed to adhere on collagen-precoated glass coverslips for an additional 1 h. After fixation, permeabilization, and blocking with 3% BSA, cells on coverslips were incubated with FITC-phalloidin. Mounted cells were analyzed and photographed under a microscope. Arrows: Stress fiber formed in (**a**) adherent cells but disappeared in (**b**) EGCG-treated cells. Some deep staining was observed in the center of EGCG-treated cells Bar: 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

EGCG treatment caused an inhibition of FAK phosphorylation at Tyr^{397} (panel a). In contrast, although ERK1/2 phosphorylation in fibroblasts were slightly increased during cell adhesion, it was not significantly inhibited by EGCG (panel b).

We next determined if actin cytoskeleton organization during cell adhesion was affected by EGCG. Immunofluorescence microscopy showed that collagen-adherent fibroblast spread well. Typical long stress fibers running across the cell body were observed (Fig. 6B, panel a, arrows). In the presence of EGCG, the changes of actin cytoskeleton were obviously noted, i.e., the disappearance of the stress fibers and the generation of protrusions at the cell periphery were observed (panel b, arrows).

EGCG Inhibited Fibroblast Migration and MMPs Activities

As described earlier, proliferation and migration of fibroblasts are proposed to be required for tumor progression. To further determine the effect of EGCG on fibroblast migration, in vitro wound closure assay and a modified Boyden chamber model were performed [Leavesley et al., 1993; Cha et al., 1996]. In the wound closure assay, scrape wounds were generated in confluent serum-free fibroblast cultures and, after extensive wash with PBS and medium, the cells were allowed to migrate the denuded area for a further 24 h (Fig. 7A, a and b). Control cells migrated into the denuded area. In the presence of EGCG, some rounded cells were observed and

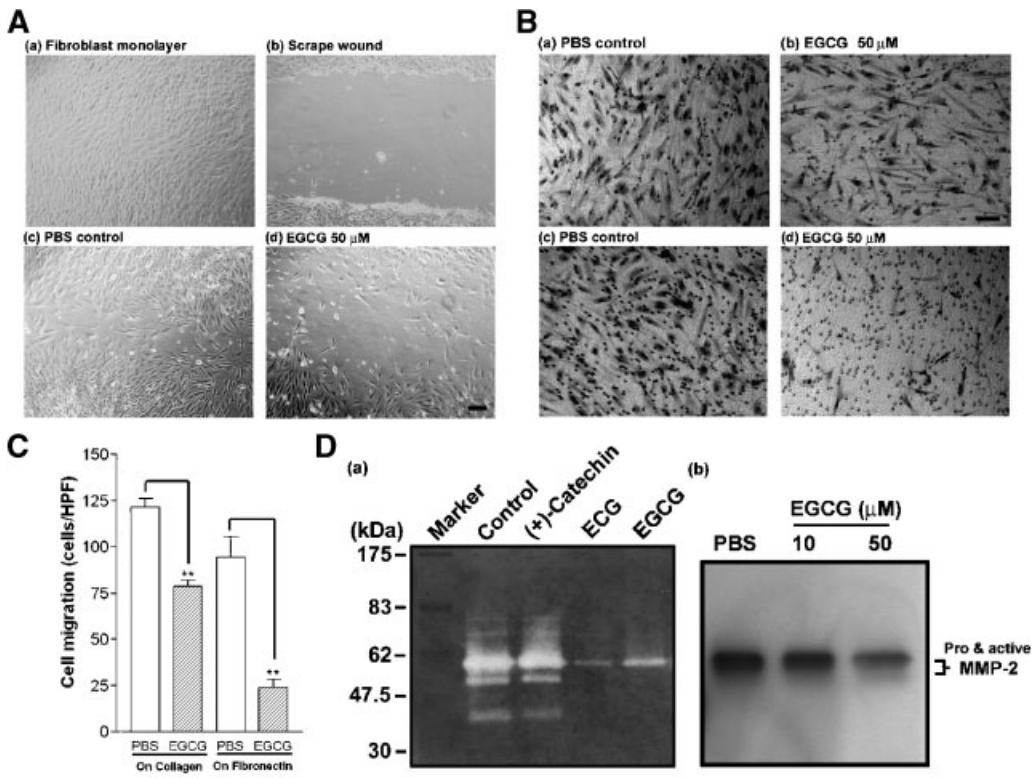

Fig. 7. Effects of EGCG on fibroblast migration, MMPs expression and activity. **A**: In vitro wound closure assay. **a**: Fibroblast monolayer cultured on 24-well plate was starved in serum-free medium. **b**: Scrape wounds were made with a tip, and after washing the cells were allowed to migrate (**c**) in the absence or (**d**) presence of EGCG. Micrographs of fixed cells at 24 h after scraping are shown. Bar: 100 μm. **B**: Transwell migration assay. Traswell inserts were coated with collagen (**a**, **b**) or fibronectin (**c**, **d**). Fibroblasts (5×10^4, 200 μl) were seeded in the upper chamber in the absence or presence of EGCG. The inserts were assembled with the lower chamber (DMEM, 600 μl) and incubated for 20 h at 37°C. After fixation and removal of nonmigrated cells, cells that migrated to the underside of filter membrane were (B) photographed and (**C**) quantified by phase-contrast light microscope under high power field (magnification, 100×). All experiments were conducted in duplicate and similar results were repeated at least three times. Data were presented as cells/HPF and were mean ± SEM (n = 10). $^*P < 0.05$ versus PBS control. **D**: (**a**) Gelatin zymographic and (**b**) Western blot analyses of MMPs in fibroblast-conditioned media. Cells cultured on collagen-precoated plates were treated with PBS (control), (+)-catechin (50 μM), ECG (50 μM), or EGCG (10 and 50 μM) at 37°C for 24 h. The media were removed, centrifuged, and then analyzed by gelatin zymography and Western blotting. Each zymograph and blot shown is the representative from three independent experiments.

migration was attenuated (c and d). The result was confirmed by the observation that fibroblast migration on collagen and fibronectin were reduced in the presence of EGCG, as analyzed under the microscope (Fig. 7B). Quantitative analysis of these migrant cells demonstrated that EGCG significantly inhibited fibroblast migration in a modified Boyden chamber model (Fig. 7C).

Since MMPs have been implicated in the remodeling of ECM and contributed in the multiple steps of tumor progression, gelatin zymography was performed to determine if EGCG affected MMPs activity. Figure 7D showed that fibroblast secreted several MMPs into culture medium. The enzymatic activities of these MMPs toward gelatin were almost abolished by the treatment of EGCG and ECG but not by (+)-catechin (panel a). We next performed Western blotting to identify these proteins and the results indicated that the cleaved bands migrating at the molecular masses of 60 and 55 kDa in non-reduced condition were pro and active MMP-2 but not MMP-1 (panel b and data not shown). EGCG inhibited MMP-2 expression in a dose-dependent manner, indicating that MMP-2 activity inhibited by EGCG is through affecting MMP-2 expression. The inhibitory effect of EGCG on MMPs activity may explain, at least in part, why EGCG inhibits fibroblast migration.

EGCG Inhibited Melanoma Cell Tube Formation in Non-Contact Cocultures

A recent study revealed that human melanoma cells form tubular structures with lumen(s) and networks when grown on three-dimensional (3-D) culture, phenomena referred to as vasculogenic mimicry [Maniotis et al., 1999; Hendrix et al., 2001]. We therefore tested whether EGCG affects melanoma cell tube formation when cocultured with fibroblasts in the modified Boyden chamber system. Figure 8 shows that melanoma cells formed a tubular structure when cocultured with fibroblasts (panel a), indicating that melanoma cells differentiated when grown on 3-D Matrigel. However, the tubular structure was disrupted when

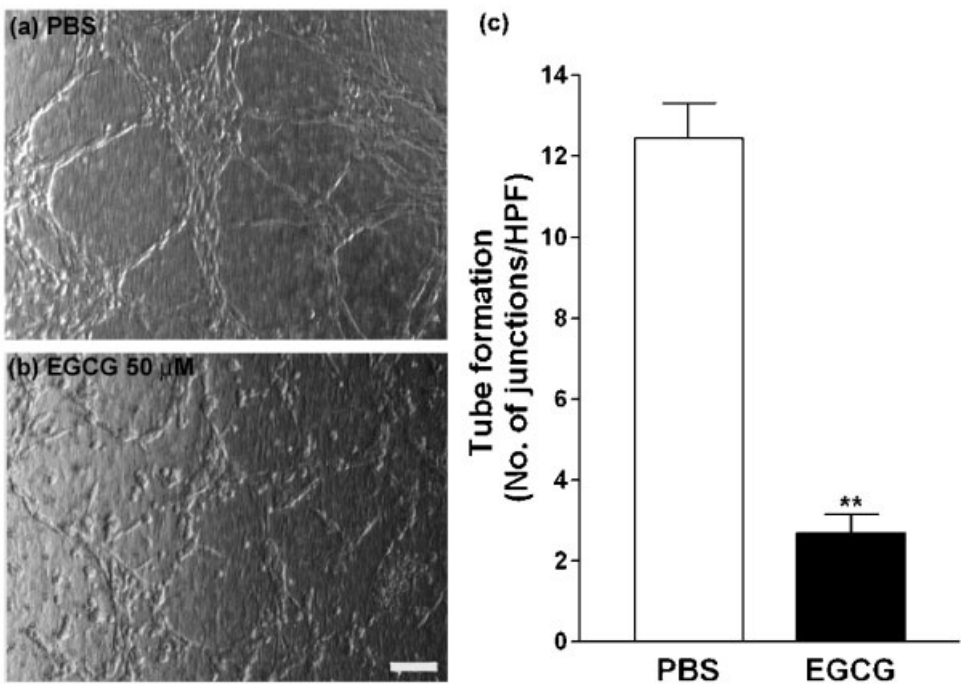

Fig. 8. EGCG inhibited the tubular structure formed by melanoma cells in non-contact coculture system. Fibroblasts cultured in the lower chamber were starved in serum-free medium and then treated with (**a**) PBS or (**b**) EGCG. After assembling of two chambers, melanoma cells (5×10^4, 200 μl) in the serum-free medium were seeded in the Transwell inserts (upper chamber) precoated with 3-D Matrigel. The two types of cells were cocultured at 37°C for 20 h and the tubular structure formed by melanoma cells in the Tranwell inserts were washed, fixed, and photographed. **c**: The degree of tube formation (no. of junctions) was quantified by counting the number of junctions per ×100 field under a phase-contrast microscope. Results were mean ± SEM (n = 6). $^{**}P < 0.01$ versus PBS control.

fibroblasts were treated with EGCG (panel b). EGCG decreased the number of junctions by 75% (panel c), indicating that EGCG interfered with melanoma cell behavior in a coculture system.

DISCUSSION

Recently, the role of the neoplastic microenvironment has become appreciated largely due to the realization that tumors are not merely masses of neoplastic cells, but instead, are complex tissue composed of both a noncellular (matrix proteins) and a cellular "diploid" component (tumor-associated fibroblasts, capillary-associated cells and inflammatory cells) [van Kempen et al., 2003]. Among the tumors, cutaneous melanoma, the incidence of which is rising, is a highly malignant tumor type and notorious for its tendency to give rise metastases. Therefore, melanoma–fibroblast interactions were focused in recent years. On the other hand, EGCG, a major polypenolic compound in green tea, has been demonstrated to have anti-tumor activity [Mukhtar and Ahmad, 2000; Kaszkin et al., 2004]. Preclinical research of EGCG is promising for cancer prevention [Moyers and Kumar, 2004]. However, there are few reports examining whether EGCG affects behavior of stromal cells such as fibroblasts. Therefore, in the present study, we determined the possible actions of EGCG on fibroblasts behaviors. We found that both EGCG and ECG, belonging to galloyl polyphenolic compounds, were able to inhibit fibroblast adhesion to collagen, fibronectin, and fibrinogen. On the contrary, another polyphenolic catechin, (+)-catechin, although prevents human keratinocytes from ultraviolet B (UVB)-induced death at the same dose (50 μM) (Submitted for publication), did not show any inhibition on fibroblast adhesion to matrix proteins (Fig. 1). This suggests that the inhibitory activity of EGCG and ECG on cell adhesion is specific for the interactions between fibroblasts and matrix proteins. Several lines of evidence indicate that the inhibitory effects of EGCG on fibroblasts were not due to its cytotoxic or pro-apoptotic activities. First, EGCG at the same concentration elicited different inhibitory effects on cell adhesion to matrix proteins (Figs. 1 and 3), suggesting its differential specificities toward matrix proteins. Second, EGCG treatment for 24 h neither caused the increase of LDH release in culture media nor decreased fibroblast's viability as determined by cytoxicity and cell viability assays, respectively (Fig. 2A). Third, suspended cells incubated with EGCG (50 μM) for 3 h did not cause any increases of dead cells and LDH release, as determined by trypan blue exclusion and cytoxicity assays, respectively (data not shown).

A specific ECM molecule can be bound by different types of integrin. Previous studies showed that integrin α2β1 is a collagen receptor favoring type I collagen, and is expressed predominantly on the basal surface of the epithelial cells [Wu and Santoro, 1994], but is also found on fibroblasts [Gardner et al., 1996]. In addition, integrin α5β1 is the major fibronectin receptor in fibroblasts [Yang et al., 1993] and integrin αvβ3 and ICAM-1 is required for human adhesion to fibrinogen [Farrell and Al-Mondhiry, 1997]. Our results showed that EGCG inhibited fibroblast adhesion to collagen, fibronectin, and fibrinogen through different action mechanisms. EGCG may interfere with fibroblast adhesion to fibronectin and fibrinogen by acting on cell integrins and/or binding to both ECM proteins (Figs. 1 and 3). Binding to the specific domain within these matrix proteins may block the specific ligation between integrin and ECM proteins. Similar results were obtained by Sazuka et al. [1998] and Suzuki and Isemura [2001]. The authors suggested that EGCG impairs melanoma cell adhesion by binding to laminin and fibronectin. However, they did not exclude the possibility that EGCG may affect integrin interaction with matrix proteins. In our study, when EGCG was incubated with collagen at the beginning and followed by a brief wash to remove unbound EGCG, the inhibitory effect of EGCG disappeared (Fig. 3B). In contrast, while cells were pretreated with EGCG and washed to remove unbound EGCG, their adhesion to collagen was compromised (Fig. 3A). The results indirectly demonstrated that EGCG did not bind to type I collagen but may affect integrin α2β1 binding. Since in cutaneous tissues, i.e., skin, type I collagen accounts for 80%–90% of all collagenous proteins [Kietly et al., 1993]. We therefore focused on how EGCG affected fibroblast adhesion to type I collagen. Flow cytometric analysis showed that the binding of mAb raised against integrin α2β1 was blocked in the presence of EGCG but not (+)-catechin (Fig. 4). This correlates with the data shown on Figures 1 and 3.

Chiarugi et al. [2003] have shown that intracellular reactive oxygen species (ROS) are generated after integrin α5β1 engagement and these oxidant intermediates are necessary for integrin signaling during fibroblast adhesion and spreading. Moreover, Honoré et al. [2003] have shown that the production of ROS increased in adenocarcinoma cell line after ligation with type IV collagen and is responsible for the control of G_1/S transition. In our study, by using DHR 123 as a probe for the detection of intracellular H_2O_2, we did not detect significant increase in H_2O_2 while fibroblast adhesion to type I collagen or fibronectin (Fig. 5 and data not shown). The results were similar by using another dye, CM-H_2DCFDA, a chloromethyl derivative of H_2DCFDA that should exhibit much better retention in live cells. However, while cells were treated with EGCG, intracellular H_2O_2 level in suspended and adherent cells decreased. The decrease of intracellular H_2O_2 level may be due to its antioxidant activity [Katiyar and Elmets, 2001; Katiyar et al., 2001]. Since the adhesion to collagen was inhibited by EGCG, we suggested that the tonic maintenance of intracellular H_2O_2 level may be required for cell adhesion.

In response to integrin engagement, several tyrosine kinases are activated, including FAK and Src. These tyrosine kinases phosphorylate substrates, leading to the activation of Rac and Cdc42. This activation finally promotes actin assembly [DeMali et al., 2003]. We demonstrated that the increase in FAK phosphorylation was inhibited in the presence of EGCG (Fig. 6). In addition, actin cytoskeleton organization, i.e., stress fibers, in adherent fibroblasts were changed upon EGCG treatment. As described earlier, Chiarugi et al. [2003] demonstrated that integrin-induced ROS are required to oxidize/inhibit low molecule weight phosphotyrosine phosphatase (LW-PTP), preventing the enzyme from dephosphorylating and inactivating FAK. Therefore, EGCG-induced decrease of intracellular H_2O_2 level may enhance LW-PTP activity, leading to FAK dephosphorylation (Fig. 6). On the other hand, EGCG may affect the expression and/or affinity of integrin α2β1 receptor, inhibiting the interaction between integrin α2β1 and collagen. It has been shown that EGCG attenuates $CD8^+$ T cell adhesion and migration by binding to CD11b (an integrin receptor that is composed of α- and β2-chains) [Kawai et al., 2004]. The authors suggested that EGCG affects CD11b expression. Although our results demonstrated that mAbs binding to integrin α2β1 was attenuated by EGCG (Fig. 4), it is still unknown whether the effect was due to the decrease of integrin expression and/or affinity. A recent study has demonstrated that EGCG can incorporate into cell membrane and reduce PDGF binding to its receptor [Weber et al., 2004]. During the preparation of samples for flow cytometric analysis, we have tried to incubate EGCG-pretreated fibroblasts with mAb in the presence of additional EGCG. However, the lost of immunofluorescence was not further enhanced (data not shown), indicating that EGCG may not bind to integrin α2β1. Since the decrease of binding is only 10%–15% of control, we suggest other action mechanism(s) of EGCG is (are) involved.

Regarding the effects of EGCG on cell migration and MMPs activity, we demonstrated that EGCG inhibited fibroblast migration and markedly inhibited MMPs activity of fibroblasts (Fig. 7). In in vitro wound closure assay, some round cells were observed in the presence of EGCG, indicating that the adhesion of migrating cells may be inhibited. Moreover, in Transwell migration model, we clearly showed that EGCG inhibited fibroblast migration toward collagen and fibronectin. Therefore, the inhibitory effect of EGCG on migration may come from its effect on fibroblast adhesion and/or on MMPs activity. Many studies have demonstrated that tumor progression required the expression of activated MMPs, which degrade extracellular matrix and regulate cell behavior [Sternlicht and Werb, 2001]. Our results demonstrated that activity and expression of the secreted MMPs, including the pro and active MMP-2, were inhibited in the presence of EGCG, indicating that EGCG may have a potential to inhibit melanoma progression. Indeed, we found that fibroblasts in the lower chamber incubated with EGCG causes the disruption of melanoma cell tubular structure in the upper chamber, as assayed in a non-contact coculture system (Fig. 8). In a recent study, Nihal et al. [2005] have demonstrated that EGCG has anti-proliferative and proapoptotic effects on human melanoma. Therefore, it is possible that EGCG not only affected fibroblasts in the lower chamber, but also melanoma cells in the upper chamber via simple diffusion. Interestingly, in response to EGCG, we found that melanoma

cells were relatively less susceptible to cell death when cocultured with fibroblasts, as determined by microscopic analysis and MTT assay (data not shown), indicating indirectly that the fibroblast–melanoma interaction occurred in the non-contact cocultures. However, EGCG still exerted inhibitory effect on melanoma cells in such coculture system, indicating that EGCG may affect tumor–stroma interaction.

In conclusion, in the present study we provided the first evidence that EGCG inhibited fibroblast adhesion and migration. The action mechanisms of EGCG were elucidated. EGCG may interfere with integrin $\alpha 2\beta 1$ interaction with type I collagen possibly through the decrease of intracellular H_2O_2 level and/or the expression or affinity of integrin $\alpha 2\beta 1$ receptor. The intracellular signaling, including FAK phosphorylation and actin cytoskeleton reorganization, was affected by EGCG. Moreover, the basal level of MMPs activity and expression was markedly inhibited by EGCG. Although previous studies demonstrated that EGCG is a potent inhibitor in affecting tumor cell behavior such as adhesion, migration, and signaling, our study provided the first evidence that EGCG also affects stromal fibroblasts and melanoma cells. The findings and concepts disclosed here provide a sound and important basis for a further exploration towards understanding tumor–stroma interaction.

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