Involvement of Impaired Interaction with β1 Integrin in Epigallocatechin Gallate-Mediated Inhibition of Fibrosarcoma HT-1080 Cell Adhesion to Fibronectin

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Epigallocatechin gallate (EGCG) is known to impair adhesion of various types of tumor cells to extracellular matrix proteins such as fibronectin and laminin by binding to fibronectin and laminin. However, it is not clear whether the direct binding of EGCG to tumor cells causes a similar impairment. In this study, we examined whether EGCG prevents tumor cells from adhering to fibronectin by binding to a fibronectin receptor, β1 integrin. Human fibrosarcoma HT-1080 cells were incubated with EGCG at various concentrations. After being washed with serum-free cell culture medium, they were plated onto fibronectin-coated wells, and the adhesion activity was examined. The results showed that the pre-treatment with EGCG inhibited cells from adhering to fibronectin in a dose-dependent manner. Cell extracts were then loaded onto an EGCG-immobilized agarose gel column and the bound fractions were examined by enzyme-linked immunoassay and Western blotting using anti-integrin β1 antibody. The results indicated that integrin β1 was bound by the column, demonstrating the interaction between integrin β1 and EGCG. These results suggest that EGCG prevents HT-1080 cells from adhering to fibronectin by impairing interaction between the cells and integrin β1.

Key words —— epigallocatechin gallate, fibronectin, cell adhesion, fibrosarcoma, collagen

INTRODUCTION

Animal studies have shown that tea and tea components have anti-cancer activities.[1–4] Green tea and black tea catechin compounds such as (–)-epigallocatechin gallate (EGCG) and theaflavin have been investigated intensively to reveal the molecular basis for their anti-tumor activities.[1–4]

Adhesive interaction between tumor cells and extracellular matrix proteins such as fibronectin, laminin, and collagens is deeply involved in tumor growth, invasion, and metastasis.[5,6] These extracellular proteins comprise the endothelial basement membrane,[7,8] and interruption of tumor cell adhesion may be effective in the prevention of blood-borne metastasis. Interactions between tumor cells and these extracellular proteins are regulated by integrins, a family of heterodimeric transmembrane receptors.[9] In tumor cells, integrins have been shown to mediate the organization of the extracellular matrix,[10] adhesion to extracellular matrix proteins,[11–13] and cell motility.[14–16]

We have reported that the binding of EGCG to Fas, presumably on the cell surface, triggers Fas-mediated apoptosis in tumor cells[17] and that EGCG impairs the adhesion of cancer cells to extracellular matrix proteins such as fibronectin and laminin by binding to fibronectin and laminin.[18,19] However, it is not clear whether the binding of EGCG to tumor cells causes an impairment of cell adhesion. In the present study, we examined whether or not EGCG inhibits the adhesion of fibrosarcoma HT-1080 cells to fibronectin by binding to the fibronectin receptor, integrin β1.

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MATERIALS AND METHODS

Chemicals ——— EGCG was obtained from Funakoshi Co. Ltd., Tokyo, Japan, and coupled to CNBr-activated Sepharose 4B at a concentration of 5 mg/ml of wet gel. Human plasma fibronectin and porcine type I collagen were from Asahi Techno Glass Co. Ltd., Tokyo, Japan. Alamar blue was a product of Alamar Biosciences, Sacramento, CA, U.S.A. The serum-free cell culture medium Cosmedium 001, a mouse anti-human integrin β1 monoclonal antibody (clone P4G11, Chemicon International, Inc., Tokyo, Japan) and a Trypan blue solution were purchased from Cosmo Bio Co. Ltd., Tokyo, Japan. Horseradish peroxidase- and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin G (IgG) antibodies were from DAKO Japan Co. Ltd., Kyoto, Japan. The peroxidase substrate solution, BM Blue POD Substrate, was from Roche Diagnostic Ltd., Tokyo, Japan.

Cell Adhesion to Fibronectin and Type I Collagen in the Presence of EGCG ——— Human fibrosarcoma HT-1080 cells were obtained from the Health Science Research Resources Bank, Osaka, Japan, and maintained in a culture medium of 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) with 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 50 μg/ml gentamycin at 37°C under 5% CO_2. Adhesion of HT-1080 cells to fibronectin- or type I collagen-coated wells in the presence of EGCG in solution was examined as follows. Sumilon plastic 48-well multidishes (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) were coated with fibronectin or type I collagen at 10 μg/ml in DMEM at 37°C for 30 min. After being washed three times with 0.2 ml of DMEM, wells were blocked by incubation with 1% bovine serum albumin in DMEM at 37°C for 30 min. After three washes with serum-free cell culture medium Cosmedium 001, wells received 0.1 ml of Cosmedium 001 with or without EGCG at various concentrations. Freshly trypsinized cells were washed three times with Cosmedium 001, 1×10^4 cells in 0.1 ml were plated onto each well, and the mixture was incubated at 37°C in a humidified CO_2 incubator. After 1 hr, wells were washed three times with 0.2 ml of Cosmedium 001 and finally received 0.1 ml of Cosmedium 001. Ten microliters of the Alamar blue solution was added to each well, and the mixture was incubated at 37°C in a CO_2 incubator. After incubation for 2 hr, fluorescence was measured with excitation at 560 nm and emission at 590 nm as described previously.[20]

Cell Adhesion to EGCG-Treated Fibronectin and Type I Collagen ——— To examine the adhesion to EGCG-treated fibronectin and EGCG-treated type I collagen, fibronectin-coated and type I collagen-coated wells were incubated with EGCG at various concentrations. An aliquot of cell suspension was added to each well, which had been washed 3 times with Cosmedium 001, and cell adhesion was determined as described above.

Adhesion of EGCG-Treated Cells to Fibronectin and Type I Collagen ——— For pre-treatment, HT-1080 cells were incubated with EGCG at various concentrations in Cosmedium 001 at 37°C for 30 min. The cells were then washed with Cosmedium 001 three times and plated onto fibronectin and type I collagen-coated wells. Their adhesion was examined as described above.

Affinity Chromatography ——— The binding between integrin β1 and EGCG was examined by affinity chromatography according to a method described previously.[17] A cell lysate in 5% Triton X-100 was loaded onto an EGCG-Sepharose 4B column. After being washed with phosphate-buffered saline (PBS), the column was eluted with PBS containing 4 M urea and 1 M NaCl. Integrin β1 in the eluates was then monitored by enzyme-linked immunoadsorbent assay (ELISA) using mouse anti-human integrin β1 antibodies and horseradish peroxidase-conjugated rabbit anti-mouse IgG antibodies essentially as described previously.[21] Peroxidase activity was determined using BM Blue as a substrate according to the manufacturer's instructions.

Electrophoresis and Western Blotting ——— To confirm the presence of integrin β1 in the EGCG-bound fraction, fractions reactive with anti-human integrin β1 antibodies were combined and examined by Western blotting. Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed according to Laemmli.[22]

After electrophoresis, proteins in gels were electrically transferred to a PVDF membrane. The membrane was incubated for 60 min with 5% defatted milk in PBS containing 0.1% Tween 20, and then treated with a 1000-fold dilution of mouse anti-human integrin β1 antibodies in 1% defatted milk dissolved in the same buffer. The membrane was washed, incubated with a 10000-fold dilution of horseradish peroxidase-conjugated second antibody and then washed and analyzed using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Tokyo, Japan).

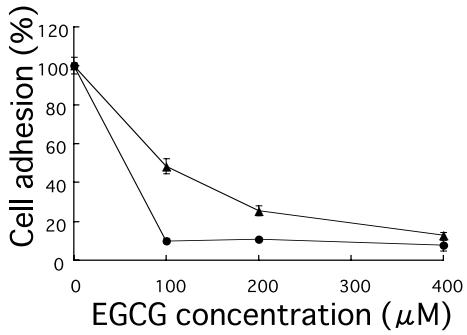

Fig. 1. Adhesion of Fibrosarcoma HT-1080 Cells to Fibronectin- and Type I Collagen-Coated Surfaces

HT-1080 cells (2×10^4 in 0.1 ml of Cosmedium 001) were cultured in the presence or absence of EGCG at various concentrations in solution in a 48-well multidish that had been coated with fibronectin (●) or type I collagen (▲) at 10 μg/ml. After 1 hr incubation, non-attached cells were removed by aspiration, and the number of attached cells was determined by Alamar blue assay. The results are expressed as the relative cell number assessed by the Alamar blue assay, where the value for the cells incubated without EGCG is taken as 100%. Data presented are averages ± S.D. from triplicate cultures.

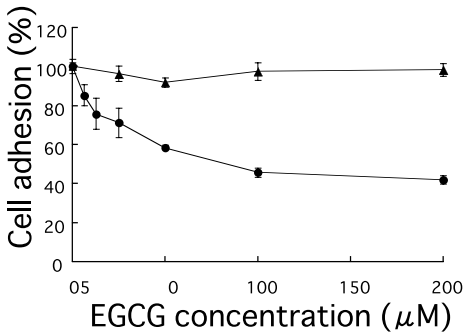

Fig. 2. Adhesion of HT-1080 Cells to EGCG-Treated Fibronectin and EGCG-Treated Type I Collagen

A multidish coated with fibronectin (●) or type I collagen (▲) at 10 μg/ml was pre-treated by incubation with EGCG at various concentrations at 37°C for 30 min. A cell suspension was added to each washed well, and attached cells were examined. Data presented are averages ± S.D. from triplicate cultures.

Flow Cytometry —— Freshly trypsinized cells were washed three times with Cosmedium 001 and 1×10^6 cells were incubated with EGCG at various concentrations in Cosmedium 001 at 37°C for 60 min. For determination of the expression of integrin β1, the cells were incubated with control mouse IgG (10 μg/ml) as a control experiment or mouse anti-human integrin β1 (1 : 100) for 60 min on ice. After the cells were washed three times with PBS containing 0.1% fetal bovine serum (FBS), they were labeled with FITC-labeled anti-mouse IgG (1 : 100) for 60 min. After three more washes with PBS containing 0.1% FBS, fluorescence staining was analyzed using an EPICS XL System II (Coulter Ltd., Tokyo, Japan).

RESULTS

Cell Adhesion to Fibronectin and to Type I Collagen in the Presence of EGCG

When the adhesion of HT-1080 cells to wells coated with fibronectin or type I collagen in the presence of EGCG in solution was examined, it was found that EGCG inhibited cell adhesion to fibronectin or type I collagen dose-dependently. EGCG inhibited cell adhesion to fibronectin more effectively than to type I collagen (Fig. 1). The results of the Trypan blue dye exclusion assay indicated that EGCG had no cytotoxic effects on HT-1080 cells under the conditions used (data not shown).

Cell Adhesion to EGCG-Treated Fibronectin and Type I Collagen

To examine the adhesion to fibronectin and type I collagen pre-treated with EGCG, wells coated with fibronectin or type I collagen were incubated with EGCG at various concentrations. A freshly prepared cell suspension was added to each well, which had been washed 3 times with Cosmedium 001, and cell adhesion was assessed. The results showed that EGCG inhibited cell adhesion to fibronectin in a dose-dependent manner (Fig. 2). By contrast, EGCG had no effect on cell adhesion to type I collagen (Fig. 2). These results suggest that EGCG impairs the adhesion of cells to fibronectin by binding to fibronectin.

Adhesion of EGCG-Treated Cells to Fibronectin and Type I Collagen

To examine the direct effects of EGCG on cells, HT-1080 cells were incubated with EGCG at various concentrations, and cell adhesion to fibronectin or type I collagen was assessed. The results indicated that adhesion of the EGCG-treated cells was inhibited in a dose-dependent manner in both cases (Fig. 3A and 3B). They suggest that the direct action of EGCG on the cells caused the inhibition of adhesion to fibronectin and type I collagen.

Affinity Chromatography

Extracts of HT-1080 cells were loaded onto an

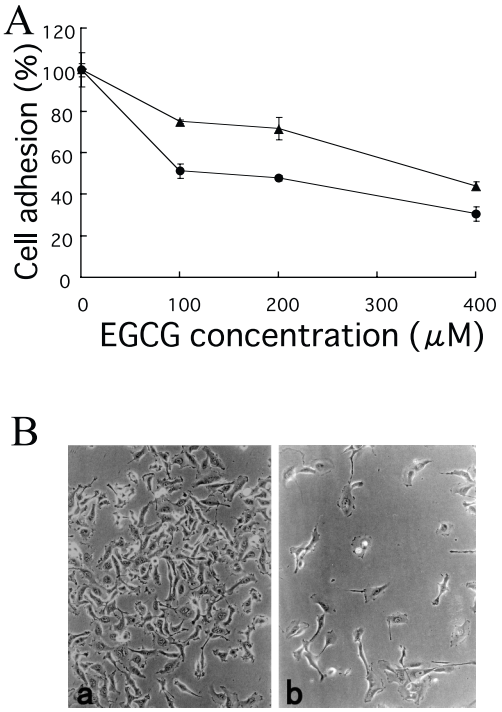

Fig. 3. Adhesion of EGCG-Treated Cells to Fibronectin and Type I Collagen

A, HT-1080 cells were incubated with EGCG at various concentrations at 37°C for 30 min. After being washed three times with Cosmedium 001, EGCG-treated cells were added to wells coated with fibronectin (●) or type I collagen (▲) at 10 μg/ml and attached cells were examined. Data presented are averages ± S.D. from triplicate cultures. B, Photographs were taken after 60 min incubation of cells treated with EGCG at 200 μM (b). Untreated cells are shown in (a). X200.

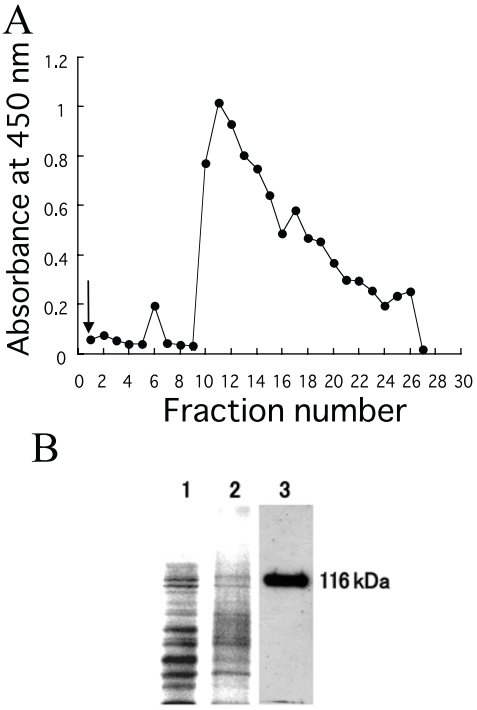

Fig. 4. Binding Affinity between Integrin β1 and EGCG

A, The supernatant of a lysate from HT-1080 cells was loaded onto an affinity column of EGCG-Sepharose 4B. After the column was washed with PBS, elution with PBS containing 4 M urea and 1 M NaCl was started at the position indicated by an arrow, and fractions of 1 ml were collected. Integrin β1 in the effluent was monitored by ELISA. Peroxidase activity was determined using BM Blue POD as a substrate according to the manufacturer's instructions. B, The cell lysate (1) and collected fractions 10–14 (2) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis and gel was silver-stained. The collected fractions 10–14 were examined by Western blotting using mouse anti-integrin β1 antibody (3).

EGCG-Sepharose column and the bound fractions were eluted with a buffer containing 4 M urea and 1 M NaCl. The effluent was monitored by ELISA using mouse anti-human integrin β1 monoclonal antibody. The results indicated that integrin β1 was bound by the column (Fig. 4A), demonstrating the interaction between integrin β1 and EGCG.

Western blotting revealed the presence of a 116-kDa protein, which was reactive to mouse anti-human integrin β1 monoclonal antibody (Fig. 4B), confirming the binding affinity between integrin β1 and EGCG.

We examined the affinity of the binding between type I collagen and EGCG using the same method, and detected type I collagen almost exclusively in EGCG-unbound fractions (data not shown), suggesting very low affinity of EGCG for type I collagen. These results demonstrated the specificity of the binding between EGCG and fibronectin.

Flow Cytometric Analysis

Results of the flow cytometric analysis of the expression of the integrin β1 subunit on the cell surface in the presence or absence of EGCG are shown in Fig. 5. EGCG had no effect on the peak of the expression. However, the cell population with low fluorescence intensity increased as the concentration of EGCG increased. These results suggest that EGCG binds directly to cell surface integrin β1 to reduce the reactivity with antibodies, and that it has no effect on integrin β1 protein expression.

DISCUSSION

Cell adhesive molecules, integrins serve as specific cell surface receptors for extracellular matrix components and contribute to the attachment, spreading, and proliferation of tumor cells.[10–16] In this study,

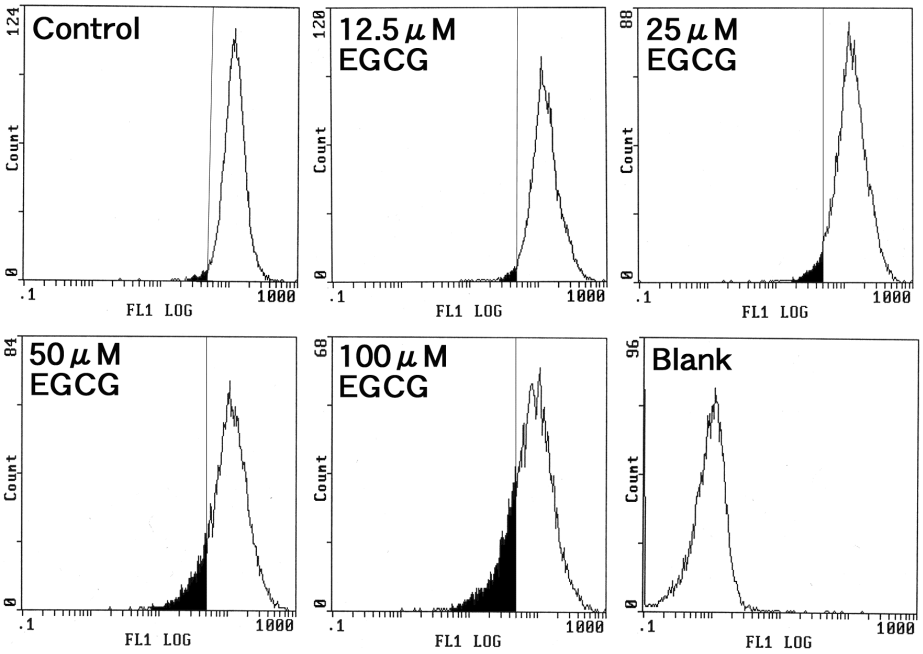

Fig. 5. Flow Cytometric Analysis for Cell Surface Integrin β1

HT-1080 cells were treated with EGCG at a concentration indicated and after a wash with PBS, integrin β1 expression on the cells was analyzed using anti-β1 integrin antibody and FITC-labeled second antibody. Control represents the result for the cells untreated with EGCG but treated with first and second antibodies. The result for the cells treated only with FITC-labeled second antibody is shown in Blank.

we investigated whether EGCG inhibits fibrosarcoma HT-1080 cells from adhering to fibronectin or type I collagen by binding to β1 integrin.

In the presence of EGCG in solution, HT-1080 cells failed to attach to fibronectin or type I collagen, especially in the former case.

When wells coated with fibronectin or type I collagen were treated with EGCG, cell adhesion to fibronectin was inhibited, but that to type I collagen was not. Previously, we reported a similar observation, that EGCG impairs the adhesion of mouse lung carcinoma 3LL cells to fibronectin by binding to fibronectin.[18] We have also reported that EGCG binds to fibronectin, fibrinogen, and histidine-rich glycoprotein in human blood plasma,[23] and demonstrated the domain-specific interaction of fibronectin with EGCG.[24] Thus, the present study provided further evidence for interaction between EGCG and fibronectin.

On the other hand, the adhesion of HT-1080 cells to type I collagen treated with EGCG was not impaired. The finding suggests that the binding of EGCG to type I collagen is too weak to have an inhibitory effect or that the binding occurs at an unidentified site which is not involved in cell adhesion. The results of affinity chromatography suggested that the former is the case. Inclusion of EGCG in the culture medium caused inhibition for the cells to attach to type I collagen, suggesting that action of EGCG on the cells is largely responsible for its inhibition of cell adhesion.

When HT-1080 cells were pre-treated with EGCG, their adhesion to fibronectin and type I collagen was inhibited. Flow cytometric analysis suggested the binding of EGCG to the cell surface, being consistent with the results of affinity chromatography demonstrating the binding between EGCG and β1 integrin. However, EGCG appeared not to affect the cell surface expression of β1 integrin. Wayner and Carter reported that a monoclonal antibody against the α subunit of β1 integrin prevented HT-1080 cells from attaching to fibronectin and to type I collagen.[25] Yamamoto *et al*. also reported that a monoclonal antibody (K20) against the β1 subunit of β1 integrin inhibited rabbit arterial smooth muscle cells from attaching to fibronectin and to type I collagen.[26] These and our findings indicate that disruption of β1 integrin either with antibodies or with EGCG results in impairment of cell adhesion.

Recently, Tachibana *et al*. reported that the cell surface 67-kDa laminin receptor, distinguishable from integrins, acts also as the receptor for anti-tu-

mor action of EGCG.[27] Thus, this is the first study to demonstrate binding between EGCG and β1 integrin. The present findings suggest that the inhibitory effect of EGCG on the binding of cancer cells to fibronectin may contribute, at least in part, to the inhibition of metastasis elicited by EGCG[28] and green tea infusion[29] as observed previously.

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