The effects of (−)-epigallocatechin gallate (EGCG) on the contraction of floating collagen gel by fibroblasts were investigated. EGCG inhibited collagen gel contraction dose-dependently. On the basis of the fact that platelet-derived growth factor (PDGF) is one of the serum components with stimulatory activity in collagen gel contraction, we examined the possibility that interaction between EGCG and PDGF may be involved in this inhibition mechanism. We confirmed this by recombinant PDGF-BB in the present system and we found that EGCG inhibited PDGF-stimulated collagen gel contraction. The results of affinity chromatography indicated that PDGF was bound by EGCG immobilized on agarose gel as detected by enzyme-linked immunosorbent and Western blotting. These findings suggest that binding of EGCG to PDGF is at least partly involved in the mechanism of inhibition of collagen gel contraction by EGCG.

Key words: collagen; fibroblasts; gel contraction; epigallocatechin gallate; platelet derived growth factor

In 1979, Bell et al.1) reported that fibroblasts incorporated in collagen gel induce a progressive contraction of the gel, resulting in the formation of a dense collagen disk with a greatly reduced diameter. This phenomenon has been considered as an in vitro equivalent of the connective tissue contraction that occurs during wound healing and other biological processes.2) It is known that collagen gel contraction is stimulated by serum. The active serum factors identified include platelet-derived growth factor (PDGF),3) transforming growth factor-β,4) endothelins,5) and thrombin.6)

We have been interested in the biological activity of green tea component catechins and have found a binding affinity between (−)-epigallocatechin gallate (EGCG) and three human plasma glycoproteins: fibronectin, fibrinogen, and histidine-rich glycoprotein.7) We assumed that the binding between EGCG and serum component(s) might impede collagen gel contraction. Recently Weber et al.8) have reported that soluble EGCG directly interacts with PDGF-BB, thereby preventing specific receptor binding to inhibit cell proliferation. Hence in this study we examined the possibility that the binding of EGCG to PDGF may be involved in the inhibition.

EGCG were obtained from Funakoshi Co., Tokyo. A trypan blue solution was purchased from Cosmo Bio Co., Tokyo. EGCG immobilized on Sepharose 4B (Amersham Pharmacia Biotech, Tokyo) was prepared as described previously.7) Acid soluble type I collagen (porcine tendon) was obtained from Nitta Gelatin Co., Osaka. Recombinant human PDGF-BB and mouse anti-PDGF-BB monoclonal antibody were obtained from Austral Biologicals Co. California, U.S.A. Goat anti-human PDGF IgG was obtained from Sigma Aldrich Japan, Co., Tokyo. The peroxidase substrate solution, Colorburst blue, was obtained from Aler CECK, Inc., Maine, U.S.A. Sigma SDS-6H was used as a molecular weight marker as described previously.7) Human fibroblasts were prepared from neonatal foreskin by the out growth method as described previously9) and maintained in DMEM supplemented with 10% FBS. For collagen gel contraction experiments, a solution of 16.7 volumes of a collagen solution at 3 mg/ml, 8.3 volumes of 3-fold concentrated DMEM, 35 volumes of DMEM, 20 volumes of FBS, and 20 volumes of the cell suspension in DMEM (2 × 10^5 cells/ml) were mixed at 4°C to give a final density of 4 × 10^4 cells/ml. Four milliliters and 1 ml of the mixed solution containing cells and collagen were incubated at 37°C in a 6 cm culture dish (Sumitomo Bakelite, Co., Tokyo) and a 12-well flat-bottomed plastic plate (Corning International, Co., Tokyo) respectively. Collagen gels formed were scraped off and diameters were measured.
When fibroblasts were cultured in three-dimensional collagen gel in DMEM containing 10% FBS for 24 h as described above, the diameter of the collagen gel decreased to about 45% of the original size (Fig. 1A). Inclusion of EGCG at 12.5 and 25 μM caused significant reduction in this contraction (Fig. 1A). Thus EGCG inhibited fibroblast-mediated collagen gel contraction in a dose-dependent manner.

EGCG has been reported to inhibit the cell growth of various cell types including virally transformed human fibroblast WI-38 cells and human epidermal carcinoma A431 cells in monolayer cultures. Since number of cells affects gel contraction, we determined cell numbers in contracted collagen gel in the presence or absence of EGCG. Cells in contracted collagen gel were incubated with 0.25% bacterial collagenase (Wako Pure Chemical Co., Tokyo) in phosphate buffered saline (PBS, pH 7.4) for 2 h at 37°C to dissolve the gel. The number of dispersed viable cells was determined by the trypan blue dye exclusion assay. The results indicated that there were no differences in cell numbers present in contracted collagen gel between the control and EGCG-treated groups during 24 h of culture (Fig. 1B). In general, cell growth within collagen gels is relatively slow. The lag time prior to the logarithmic phase growth of fibroblasts is reported to be 48 h in collagen gel (1 mg/ml), in contrast to 24 h on a plastic dish. Chen et al. have reported that EGCG at 40 μM had no or little effect on the cell growth of human normal fibroblast cells. The ineffectiveness we observed of EGCG on proliferation of cells in collagen gels is consistent with these data. These results suggest that the inhibitory effect of EGCG on collagen gel contraction was not due to inhibition of cell growth.

PDGF is known to be one of the serum factors that can stimulate the contraction of floating collagen gels. Hence the binding interaction between EGCG and PDGF might explain the inhibition of collagen contraction mediated by fibroblasts cultured in serum-containing medium. To test this possibility, fibroblasts (2 × 10^3 cells/ml) were cultured in collagen gel in DMEM containing PDGF-BB (100 ng/ml) and collagen gel contraction was determined. The results showed the stimulatory activity of PDGF (Fig. 2). When EGCG at 6.25–25 μM was present, PDGF-stimulated, fibroblast-mediated collagen gel contraction was inhibited (Fig. 2), indicating that EGCG inhibits PDGF-stimulated collagen gel contraction. Collagen gel contracted even when PDGF was added to the culture medium. The results suggest that EGCG inhibits PDGF-stimulated collagen gel contraction by blocking the binding interaction between EGCG and PDGF.

**Fig. 1.** Effects of EGCG on Fibroblast-mediated Collagen Gel Contraction and Fibroblast Cell Growth. (A) Fibroblasts (4 × 10^4 cells/ml) were cultured in collagen gels in the presence or absence of EGCG as indicated. The diameter of the dish was 52 mm. The data represent the percentage of the gel diameter relative to the original diameter and the bars with a different mark represent statistically significant differences (p < 0.05, Student’s t-test). (B) Effects of EGCG on cell growth in collagen gels. Fibroblasts in DMEM containing 10% FBS with or without EGCG were plated at an initial density of 4 × 10^4 cells/ml (1.6 × 10^5 cells/dish). Data are expressed in comparison with cell numbers cultured without EGCG (100%) and bars with the same mark represent no significant statistical difference.

**Fig. 2.** Inhibition of PDGF-Stimulated Collagen Gel Contraction by EGCG. EGCG at the concentrations indicated was added to a culture of fibroblasts (2 × 10^3 cells/ml) in floating collagen gel with or without PDGF (100 ng/ml). After 3 h (A) and 24 h (B) of culture, the diameter of the collagen gel was measured. The diameter of the dish was 22 mm. The bars with a different mark represent statistically significant differences (p < 0.05, Student’s t-test).
fibroblasts were cultured in serum-free DMEM (Fig. 2). This finding suggests that neonatal foreskin fibroblasts themselves can produce gel contraction-promoting factors such as PDGF and TGF-β, since previous studies showed production of PDGF by rat lung fibroblasts and TGF-β by human dermal and rat lung fibroblasts. EGCG appeared to cancel the collagen gel contraction-promoting activities of these endogeneous growth factors (Fig. 2).

To examine the possible binding of EGCG to PDGF in this inhibition, human recombinant PDGF-BB was loaded onto an EGCG-Sepharose column and the bound fractions were eluted with a buffer containing 4 M urea and 1 M NaCl. The effluents were monitored by enzyme-linked immunoassay using goat anti-human PDGF polyclonal antibodies and peroxidase-conjugated anti-goat IgG essentially according to the method described previously. The results indicated that PDGF-BB was retained by the column (Fig. 3A), indicating a binding interaction between PDGF-BB and EGCG. Similar results were obtained in the experiments using mouse anti-human PDGF-BB monoclonal antibody (data not shown).

To confirm the presence of PDGF-BB in the EGCG-bound fractions, Western blotting analysis was performed. Electrophoretically separated proteins were blotted onto nitrocellulose membrane and analyzed using an ECL (enhanced chemiluminescence) system (Amersham Pharmacia Biotech). The results indicated that a 29-kDa protein in EGCG-bound fractions was reactive to goat anti-human PDGF antibodies (Fig. 3B).

Previously, we reported that bovine serum albumin is detected in EGCG-unbound fractions almost exclusively when subjected to affinity chromatography with an EGCG-Sepharose column. These results indicated the specificity of the binding interaction between EGCG and PDGF. This finding is consistent with that described by Weber et al., who found direct binding between PDGF-BB and EGCG. Hence we propose that binding of EGCG to PDGF is involved in the mechanism by which EGCG inhibits fibroblast-stimulated collagen gel contraction. But it is possible that other interactions are also involved in the mechanism, since EGCG can interact with various proteins such as fibronectin, fibrinogen, and histidine-rich glycoprotein, and since various serum factors can stimulate collagen gel contraction.

A cup of green tea contains approximately 200 mg EGCG, and after drinking two to three cups of tea the concentration of EGCG in the plasma is 0.3–4 μM. Thus, the serum concentration of EGCG may be too low to exhibit biological activity on fibroblast-mediated collagen gel contraction. But it is possible that catechins accumulate in tissues, resulting in locally and temporarily high concentrations of catechins. If so, green tea and EGCG might serve as drugs in the treatment of pathological contraction of scars, since PDGF can regulate the contraction of scars or granulation tissue during the wound healing processes, as proposed for genistein.

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References


