Apoptosis induction by epigallocatechin gallate involves its binding to Fas.

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Summary

Epigallocatechin gallate (EGCG) is known to induce apoptosis in various types of tumor cells, but the precise mechanism by which EGCG induces apoptosis remains to be elucidated. The Fas-Fas ligand system is one of the major pathway operating in the apoptotic cascade. The aim of this study was to examine the possibility that EGCG-binding to Fas triggers the Fas mediated apoptosis. The EGCG treatment of human monocytic leukemia U937 cells resulted in elevation of caspase 8 activity and fragmentation of caspase 8. The DNA ladder formation caused by the EGCG treatment was inhibited by the caspase 8 inhibitor. These findings suggested the involvement of the Fas-mediated cascade in the EGCG-induced apoptosis in U937 cells. Affinity chromatography revealed the binding between EGCG and Fas. Thus, the results suggest that EGCG-binding to Fas, presumably on the cell surface, treggers the Fas mediated apoptosis in U937 cells.

Key words
Apoptosis, Epigallocatechin Gallate (EGCG), Fas, U937

Introduction

Many 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 most intensively to reveal the molecular basis for their anti-tumor activities (1-4). EGCG, a major constituent of green tea, is known to induce apoptosis in various types of tumor cells (4-14). The fact that tumor cells are much more sensitive to apoptosis induction by EGCG than the normal counterparts (6, 7) offers merit in its potential usage as a chemopreventive agent. However, the precise mechanism by which EGCG induces apoptosis remains to be elucidated, although some proposals have already been made. These include inhibition of transcription factor NF-κB (2, 12), activation of the tumor necrosis factor α-mediated signaling pathway (8), and cell cycle arrest at a G0/G1 (4, 13) or G2/M phase (8).

We have demonstrated the specific binding of EGCG to three plasma glycoproteins, fibronectin, fibrinogen and histidine-rich glycoprotein (15), and matrix metalloproteases (16) from the tumor cell culture. The Fas (also known as APO-1 or CD95)-Fas ligand system is one of the major pathways operating in the apoptotic cascade (17, 18). It is known that some antibodies against Fas (antagonistic antibodies) can inhibit the apoptotic process, while the others (agonistic antibodies) trigger the process (17, 19-22).

Thus, the stimulation of Fas by an agent other than physiological Fas ligand could also be a trigger for apoptosis. In the present study, we hypothesized that the binding of EGCG to Fas might trigger the cascade of the Fas-mediated apoptosis.

Materials and Methods

Cell culture and reagents. Human U937 cells were obtained from the Health Service Research Resources Bank, Osaka, Japan, and maintained in a culture medium of 10% fetal bovine serum in an RPMI 1640 medium with 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 50 μg/ml gentamycin at 37℃ under 5% CO_2. EGCG was obtained from Funakoshi Co. Ltd., Tokyo, Japan. EGCG-Sepharose 4B was prepared as described previously (15). Monoclonal anti-caspase 8 antibody 5F7 was obtained from Medical and Biological Laboratories, Co., Ltd., Nagoya, Japan. Rabbit anti-Fas antibody was from Wako Pure Chemical Industries, Ltd.,

Osaka, Japan, and monoclonal anti-Fas antibodies UB2 and SM1/1 (a product of Chemicon International Inc.) were from Medical and Biological Laboratories, Co., Ltd., and Cosmo Bio Co., Ltd., Tokyo, Japan, respectively. Horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) and peroxidase-conjugated sheep anti-mouse IgG were from Amersham Pharmacia Biotech, Tokyo, Japan, and the peroxidase substrate solution, BM Blue, was a product of Roche Diagnostics K. K., Tokyo, Japan. Alkaline phosphatase-conjugated goat anti-rabbit IgG and anti-mouse IgG were from Promega K. K., Tokyo, Japan.

Induction of DNA fragmentation by EGCG. U937 cells were incubated in a culture medium in the presence or absence of EGCG for 16 h at 37℃ in a CO_2 incubator. For DNA fragmentation analysis, $5X10^5$ cells were pelleted by centrifugation and DNA was isolated from the cell pellets as described by Sellins and Cohen (23). DNA was subjected to electrophoresis in 2% agarose gels, stained with SYBR Green I, and imaged using FluorImager (Molecular Dynamics Japan, Inc., Tokyo, Japan) as described previously (14, 15). To examine whether caspase 8 is involved in the DNA fragmentation caused by EGCG, the cells were incubated in the presence of EGCG at 400 μM with the caspase 8 inhibitor acetyl-IETD-CHO (24) (Medical and Biological Laboratories, Co., Ltd.) at 200 μM at 37℃ for 6 h, and DNA fragmentation was examined.

Caspase 8 activity. Caspase 8 activity was determined using an FLICE/caspase 8 fluorometric assay kit containing a fluorogenic substrate, IETD-7-amino-4-trifluoromethyl coumarin (AFC), (Medical and Biological Laboratories, Co., Ltd.) according to the manufacturer's instruction. The U937 cells incubated with EGCG at various concentrations or for various time periods were washed with phosphate-buffered saline (PBS), and lysed with the cell lysis buffer included in the kit. Fluorescence was measured at 505 nm with excitation at 400 nm.

Western blotting of caspase 8. The U937 cells incubated with EGCG for various time periods were heated at 100℃for 2 min in the sample buffer for SDS-polyacrylamide gel electrophoresis and examined by the Western blotting method. The PVDF membrane with blotted proteins was probed with anti-caspase 8 antibodies and alkaline phosphatase-conjugated second antibodies essentially according to the method described previously (25).

Affinity chromatography. The binding between Fas and EGCG was examined by affinity chromatography as follows. A cell lysate in 5% Triton X-100 from U937 cells was loaded onto an EGCG-Sepharose 4B column. After being washed with PBS, the column was eluted with PBS containing 4 M urea and 1 M NaCl. Fas in the effluents was then monitored by enzyme-linked immunoassay using anti-Fas antibodies and horseradish peroxidase-conjugated second antibodies essentially according to the method described previously (25). Peroxidase activity was detected using the substrate, BM Blue, according to the manufacturer's instruction. To confirm the presence of Fas in the EGCG-bound fractions, fractions reactive with anti-Fas antibodies were combined and concentrated 10-fold using Centricon (Millipore, Tokyo, Japan) and examined by Western blotting. The PVDF membrane with blotted antigen was probed with anti-Fas antibodies and alkaline phosphatase-conjugated second antibodies.

Flow cytometry. U937 cells were incubated with EGCG at various concentrations for 1 h and analyzed using an EPICS XL System II (Coulter Ltd., Tokyo, Japan).

Results and Discussion

Our and other works have shown that EGCG induces apoptosis in several tumor cell lines. The present findings showed that the ladder formation, one of the characteristic features of apoptosis (23), was inhibited by the caspase 8 inhibitor (Fig.1), suggesting the involvement of Fas-mediated cascade in the EGCG-induced apoptosis in U937 cells. Two lines of evidence supported this suggestion. When U937 cells were incubated with EGCG, caspase 8 activity was found to increase concentration- and time-dependently (Fig. 2). When EGCG-treated cells were subjected to the Western blotting analysis using anti-caspase 8 antibody, the results demonstrated the time-dependent activation of pro-caspase 8 as evidenced by the time-dependent

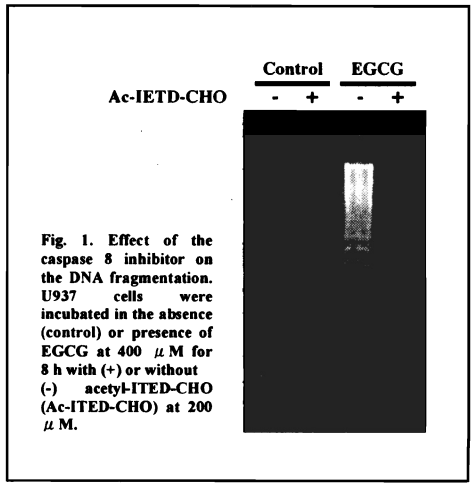

Fig. 1. Effect of the caspase 8 inhibitor on the DNA fragmentation. U937 cells were incubated in the absence (control) or presence of EGCG at 400 μM for 8 h with (+) or without (-) acetyl-ITED-CHO (Ac-ITED-CHO) at 200 μM.

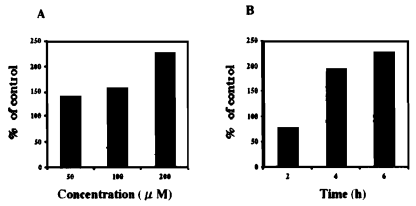

Fig. 2. Activation of caspase 8 upon treatment with EGCG. U937 cells were incubated with EGCG at various concentrations (A) for 6 h or EGCG at 200 μ M for various time periods (B). Caspase 8 activity was determined using the fluorogenic substrate ITED-AFC and is expressed as the fluorescence value relative to that for untreated cells (control).

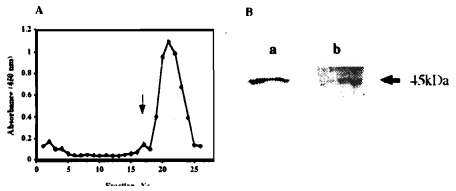

Fig. 3. Binding of Fas to EGCG immobilized on agarose. A, The supernatant of a cell lysate from U937 cells was loaded onto an affinity column of EGCG-Sepharose 4B (2 ml). After the column was washed with PBS, elution with PBS containing 4 M urea and 1 M NaCl was started as indicated by an arrow, and fractions of 1 ml were collected. Fas in the effluents was monitored by enzyme-linked immunoassay using rabbit anti-Fas antibody and peroxidase-conjugated anti-rabbit IgG. B, Fractions 20-22 were collected, concentrated and subjected to Western blotting analysis using rabbit anti-Fas antibody (a) or monoclonal antibody UB2 (b) in combination with corresponding alkaline phosphatase-conjugated second antibody and the nitrotetrazolium blue substrate.

appearance of processed bands of 45-, 43- and 18-kDa.

Flow cytometric analysis suggested the binding of EGCG to the cell surfaces, although we cannot exclude the possibility that EGCG was simply incorporated into the cell membrane, since EGCG is known to fluoresce when incorporated into liposome (26). Okabe *et al.* have already reported the possible binding of EGCG to the cell membrane of various cancer cells (27).

Agonistic anti-Fas antibodies have been shown to induce apoptosis in U937 cells (20,21), indicating the expression of Fas in these cells. The extracts of U937 cells were loaded onto an EGCG-Sepharose column and the fraction eluted with the buffer containing 4M urea and 1M Nacl were monitored by the enzyme-linked immunoassay using rabbit anti-Fas antibody. The results indicated that Fas was bound by the column (Fig.3A), demonstrating the binding interaction between Fas and EGCG. Further, we confirmd the binding between EGCG and Fas by Western blotting analysis (Fig.3B). Fas-mediated apoptosis represents the major pathway of apoptosis and involves caspase 8 activation at an initial stage (17, 18).

On the basis of these findings, it is suggested that EGCG-binding to Fas, presumably on the cell surface, triggers the Fas-mediated apoptosis in U937 cells. However, there is a possibility that the other mechanisms may be operative at the same time, since a portion of added EGCG may enter into the cells to exert its activities within the cell (28).

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