Synergistic Effect of Green Tea Catechins on Cell Growth and Apoptosis Induction in Gastric Carcinoma Cells

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(−)-Epigallocatechin gallate (EGCG), a major component of green tea catechins, is known to inhibit cell growth and to induce apoptosis in a variety of cultured cells. We examined effects of green tea catechins in cultured cells derived from human gastric carcinoma. The proliferation of four cell lines (MKN-1, MKN-45, MKN-74 and KATO-III) was inhibited with EGCG in a dose-dependent manner. The growth of MKN-45 cells was most efficiently inhibited by the treatment (IC$_{50}$: 40 μM EGCG) among the four cell lines, while KATO-III cells were most insensitive (IC$_{50}$: 80—150 μM) to the EGCG treatment. In addition, (−)-epicatechin (EC) had a major synergistic effect on the induction of apoptosis in MKN-45 cells treated with EGCG; however it had little effect on the inhibition of cell growth induced by EGCG. To study the molecular mechanisms behind the induction of apoptosis by EGCG, the activity of caspases in MKN-45 cells treated with EGCG was examined. Activity levels of caspases-3, -8 and -9 were elevated in EGCG-treated cells, suggesting that these caspases are involved in the apoptosis induced by EGCG. Furthermore, the synergistic effect of EC with EGCG on the induction of apoptosis was specifically canceled by catalase treatment, suggesting that the synergism involves the extracellular production of reactive oxygen species.

Key word   apoptosis; (−)-epigallocatechin gallate (EGCG); gastric cancer; growth inhibition; synergistic effect

Green tea is widely consumed in Japan and China and recent studies have shown that green tea and its component polyphenol have a chemopreventing effect on models of cancer *in vitro* and *in vivo*.[1] (−)-Epigallocatechin-3-gallate (EGCG) is a major component of polyphenols in green tea and is reported to inhibit the proliferation and to induce apoptosis in cancer cells.[2] A typical cup of green tea contains 100—150 mg of catechins, including 8% (−)-epicatechin (EC), 15% (−)-epigallocatechin (EGC), 15% (−)-epicatechin-3-gallate (ECG), and 50% EGCG.[3] Among these catechins, EGCG is the most potent component for inhibition of the growth of cancer cells and induction of apoptosis.[4] The molecular mechanism of the chemopreventing effect of green tea can be accounted for by the fact that green tea catechins including EGCG have an inhibitory effect on the growth of cancer cells; however, the precise mechanism of growth inhibition of catechins remains to be elucidated.[5]

Apoptosis is a programmed cell death induced by extracellular stimuli or internal signaling pathways during such processes as embryogenesis and organ development. Signaling pathways for apoptosis induced by extracellular stimuli include two major cascades; one triggered by the binding of a signaling factor such as FAS ligand to the cognate death receptor and including the activation of caspase-8 followed by caspase-3,[6,7] the other initiated by stress or toxic stimuli whereby the induced signal for apoptosis is transduced through activation of JNK/p38 and a mitochondrial pathway which includes activation of caspase-9 followed by caspase-3.[8] A recent study reported that oxidative stress induced by EGCG is a major effector inducing apoptosis through the JNK/p38 pathway,[9] whereas, Hayakawa *et al*. reported that EGCG binds to specific receptor proteins including FAS and induces apoptosis in leukemia U937 cells.[10]

In this study, we examined the effect of green tea catechins on a variety of cell lines established from gastric carcinoma, focusing on the chemopreventing effect of green tea as a functional food. Four cell lines established from gastric carcinoma cells in various states were treated with tea catechins and the inhibition of cell growth and induction of apoptosis were examined. The results suggested that the cell lines differed in their susceptibility to EGCG treatment: MKN-45, established from a poorly differentiated tubular adenocarcinoma, was the most sensitive to the EGCG treatment. To evaluate the chemopreventing effect of green tea as a beverage, the synergistic effect of catechins included in the tea was examined. EC had almost no effect on the cell growth or induction of apoptosis. However, a significant synergistic effect on the induction of apoptosis was observed when EC was combined with other catechins. Furthermore, we found that catalase specifically canceled the synergistic effect of EC and EGCG, suggesting that the synergism involved production of reactive oxidative species, such as hydrogen peroxide.

MATERIALS AND METHODS

Chemicals and Enzymes   EGCG, EGC, ECG, EC, and (+)-catechin (C) were purchased from Mitsui Norin Co. Ltd. (Tokyo, Japan); Catalase (C1345, 2350 units/mg) from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.); Proteinase K from Wako Pure Chemical Ind. (Osaka, Japan); Eagle's minimum essential medium (MEM) and RPMI1640 medium from Nissui Seiyaku (Tokyo, Japan); and fetal bovine serum (FBS) from Moregate Laboratories (Melbourne, Australia). Ribonuclease A was a product of Worthington Biochemical Co. Monoclonal antibody against caspace-3 was a product of Immunotech (Marseille, France; Cat. No. 2120). Monoclonal antibody against caspase-8 and caspase-9 were purchased from MBL (Nagoya, Japan; Cat. No. M054—3 and M032—3).

Cell Culture Conditions   Cultured cells established

from gastric carcinoma, MKN-1, MKN-45, MKN-74, and KATO-III, were provided by RIKEN Cell Bank, RIKEN BioResource Center (Tsukuba, Japan). All cell lines were grown in RPMI 1640 medium supplemented with 10% FBS, 100 μg/ml ampicillin and 100 μg/ml kanamycin. HeLa cells provided by RIKEN Cell Bank, RIKEN BioResource Center (Tsukuba, Japan) and grown in MEM supplemented with 10% FBS, 100 μg/ml streptomycin and 60 μg/ml kanamycin. For catechin treatment, about 1×10^5 cells per milliliter of each cell line were plated into tissue culture dishes and precultured for one day. Then, the cells were treated with catechins for an appropriate period. The culture medium was renewed every three days during the EGCG treatment. Cell growth was measured by counting viable cells after staining with trypan blue.

Detection of Apoptosis by DNA Ladder Assay Cultured cells treated with EGCG were harvested and lysed in cell lysis buffer containing 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100. After incubation on ice for 10 min, cells were lysed by vortexing for 10 s and the lysate was collected by centrifugation at 10000$\times$**g** for 20 min at 4 °C. Cell lysates were incubated with 400 μg/ml RNase A for 1 h at 37 °C, then proteinase K was added at a final concentration of 400 μg/ml and incubated at 37 °C for 1 h. The sample was subjected to electrophoresis on a 2% agarose gel in 1$\times$TAE buffer containing 40 mM Tris–acetic acid (pH 8.1) and 2 mM EDTA, and DNA bands on the gel were visualized by ethidium bromide staining.

Detection of Apoptosis by Enzyme-Linked Immunoassay MKN-45 cells treated with catechins were harvested, cytosolic extracts were prepared from each sample, and DNA fragmentation was measured by determination of cytosolic oligonucleosome-bound DNA using a Cell Death Detection ELISA (Boehringer Mannheim, Mannheim, Germany), according to the procedure described by the manufacturer. Amounts of oligonucleosome released into the cytoplasm were measured by absorbance at 415 nm using a sandwich enzyme-linked immunoassay. To examine the effects of catalase on catechin-induced apoptosis, catalase was added to the medium at a final concentration of 50—100 U/ml. MKN-45 cells were incubated for 30 min with catalase before the addition of catechins. After 24 h of treatment with catechins, the extent of induction of apoptosis was measured by the enzyme-linked immunoassay.

Determination of Activity of Caspases The activity of caspases-3, -8 and -9 was measured using Caspase/FRICE Fluorometric Protease Assay Kit (BioVision, CA) designed for each caspase. MKN-45 cells (1.5×10^5) treated with 100 μM EGCG were resuspended with 0.2 ml of extraction buffer containing 50 mM Tris–HCl (pH 7.4), 0.5% NP-40, 200 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM DTT, 1 mM PMSF, 0.5 mM TPCK, 25 μM TLCK, and 2 μg/ml pepstatin A. After incubation on ice for 30 min, supernatants were collected as cell lysates after centrifugation at 10000$\times$**g** for 30 min at 4 °C. Cell lysates were resuspended in 50 μl of cell lysis buffer contained in the assay kit, then, 50 μl of 2$\times$reaction buffer and 5 μl of the 1 mM AFC-conjugated substrate were added. The mixture was incubated at 37 °C for 2 h. Caspase activity was determined by measuring fluorescence under UV light at 365 nm. The image of each sample on a 96-well microtiter plate was digitized with the FAS-III image

capture system (Toyobo Inc., Osaka, Japan) and the intensity of fluorescence of each sample was measured with NIH-Image data analyzing software.

Detection of Activated Caspase by Western Blotting The cell extracts prepared from MKN-45 cells treated with 200 μM EGCG were mixed with 2$\times$SDS sample buffer containing 100 mM Tris–HCl, 20% glycerol, 2% SDS, 0.01 mg/ml bromophenol blue, and 100 mM dithiothreitol. The mixture were incubated at 95 °C for 4 min and subjected to electrophoresis through a 13% SDS-polyacrylamide gel. After the electrophoresis, proteins on the gel were electrotransferred onto Hybond-P membranes (Amersham, Buckinghamshire, U.K.) with transfer buffer containing 25 mM Tris–HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were incubated in blocking buffer containing 20 mM Tris–HCl (pH 7.6), 125 mM NaCl, 0.1% Tween-20, and 5% skim milk (Wako Pure Chemical Ind., Osaka, Japan) for 1 h. The membranes were then incubated with an antibody against each caspase in PBS supplemented with 0.05% Tween-20 and 5% skim milk at room temperature for 1 h. The antibodies on the membrane were detected using ECL Western blotting detection system (Amersham, Buckinghamshire, U.K.).

RESULTS

Treatment with Tea Catechins Causes Dose-Dependent Inhibition of Cell Growth in Cultured Cells Established from Gastric Carcinoma We first examined the effect of EGCG on the growth of cell lines established from gastric carcinoma. As shown in Fig. 1, growth of MKN-1, MKN-45, MKN-74, and KATO-III cells was inhibited in a dose-dependent manner and EGCG had the greatest inhibitory effect on MKN-45 cells. The median inhibitory concentration (IC$_{50}$) of EGCG for the growth inhibition in MKN-45 cells is about 40 μM, whereas the IC$_{50}$ for KATO-III cells, which are most insensitive to the EGCG treatment, is about 80—150 μM, as calculated from the results shown in Figs. 1 and 2. Then, we examined the growth inhibitory activity of tea polyphenols other than EGCG using MKN-45 cells and KATO-III cells, as well as HeLa cells as a control. As shown in Fig. 2, EC had almost no effect on cell growth, while EGCG, ECG and EGC inhibited cell growth to almost same extent in each cell line in a dose-dependent manner.
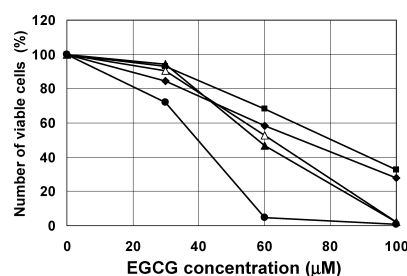

Fig. 1. Growth Inhibition of a Variety of Cancer Cells Induced by EGCG Treatment

Cultured cells established from gastric carcinoma [KATO-III (■), MKN-1 (△), MKN-45 (●), MKN-74 (▲)] and HeLa cells (◆) were treated with EGCG at various concentrations for 48 h. After incubation, viable cells were counted and the cell number of each cell line is indicated relative to the number of cells cultured under the same conditions without EGCG treatment.
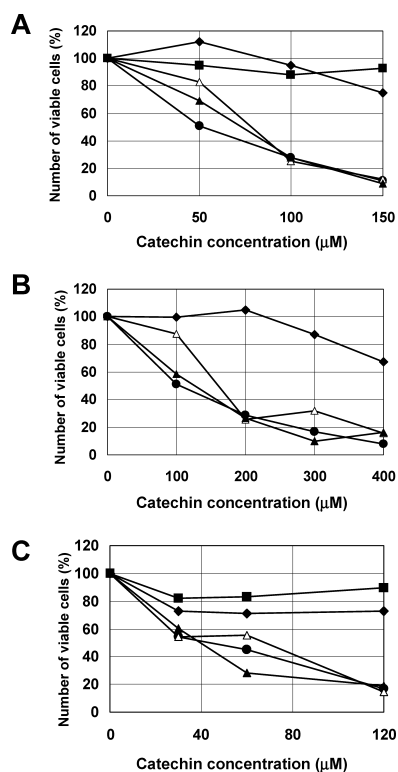
Fig. 2. Growth Inhibition of HeLa, MKN-45 and KATO-III Cells by Catechin Treatment

HeLa cells (panel A), KATO-III cells (panel B) and MKN-45 cells (panel C) were treated with C (■), EC (◆), ECG (▲), EGC (△) or EGCG (●) at the indicated concentrations for 48 h and viable cells were enumerated. The number of viable cells of each sample is indicated relative to the number of cells cultured under the same conditions without catechin treatment.
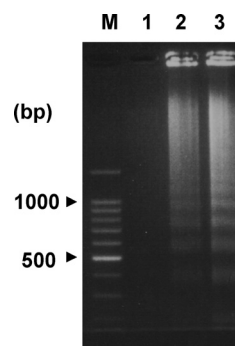

Fig. 3. Apoptosis of MNK-45 Cells Induced by EGCG Treatment

MKN-45 cells were cultured in complete media without EGCG (lane 1) or with 100 μM EGCG for 24 (lane 2) and 48 h (lane 3). Cell extracts were prepared and subjected to a DNA ladder assay as described in Materials and Methods. The sizes of the 100 bp DNA marker (lane M) are indicated on the left.
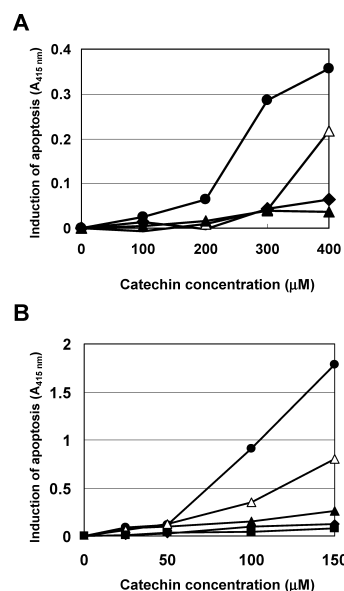

Fig. 4. Apoptosis of KATO-III Cells and MNK-45 Cells Induced by EGCG Treatment

KATO-III (panel A) and MKN-45 (panel B) cells were treated with EGCG (●), ECG (▲), EGC (△), EC (◆) or C (■) at the indicated concentration for 48 h. Cell lysates were prepared and subjected to the enzyme-linked immunoassay for the quantitative analysis of induction of apoptosis as described in Materials and Methods.

Apoptosis Was Induced by Polyphenol Treatment in the Cell Lines Established from Gastric Carcinoma Apoptosis is characterized as a physiological cell death accompanied by chromatin condensation, membrane blebbing, and the internucleosomal degradation of DNA. Nucleosomal DNA ladders which result from this internucleosomal degradation can be observed on agarose gel electrophoresis as a typical feature of apoptosis. To examine the induction of apoptosis in MKN-45 cells by EGCG treatment, the cells were treated with 100 μM EGCG for 24 h or 48 h. A nucleosomal DNA ladder formation was observed. The result shown in Fig. 3 suggested that the apoptosis was induced by the treatment with EGCG. Next, we compared effects of four tea catechins, EGCG, EGC, ECG, and EC, on the induction of apoptosis using MKN-45 cells and KATO-III cells. For MKN-45 cells, the effect of C on the induction of apoptosis was also examined. As shown in Fig. 4, EGCG was most effective in inducing apoptosis both in MKN-45 cells and in KATO-III cells and EGC had a weak activity for apoptosis induction. On the other hand, ECG, EC and C had almost no effect on the induction of apoptosis in these cell lines. These results also suggested that MKN-45 cells were more sensitive to EGCG or EGC treatment for the induction of apoptosis than KATO-III cells; however, in both cell lines, a higher concentration of EGCG or EGC was needed for induction of apoptosis compared with the concentration of EGCG or EGC for growth inhibition.

Synergistic Effect of EC and Other Catechins on

Growth Inhibition and Induction of Apoptosis in MKN-45 Cells Green tea contains four kinds of catechins as major polyphenol components. Examining the effect of the combination of catechins on cell growth is important to evaluate green tea as a cancer preventive beverage. As shown in Figs. 2 and 4, EC had a weak effect on growth inhibition and apoptosis induction even in MKN-45 cells, which were most susceptible to EGCG treatment among the cell lines we used. However, EC is reported to have a synergistic effect on the induction of apoptosis in PC9 cells when combined with other catechins.[11] To examine the synergistic effect of EC and EGCG on growth inhibition and induction of apoptosis, KATO-III cells and MKN-45 cells were incubated with various concentrations of EC in the absence or presence of EGCG. The results suggested that EC had almost no effect on the growth inhibition induced by EGCG in either MKN-45 cells or KATO-III cells (Fig. 5). In contrast, EC enhanced the induction of apoptosis by EGCG treatment synergisti-
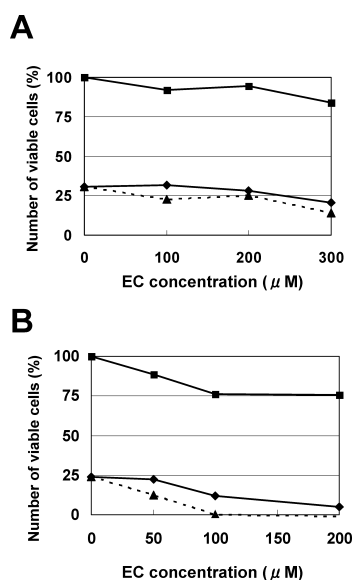
A

B

Fig. 5. Cell Growth Inhibition Induced with a Combination of EC and EGCG in KATO-III Cells and MKN-45 Cells

KATO-III cells (panel A) and MKN-45 cells (panel B) were treated with EC at the indicated concentration with 100 μM EGCG (◆) or without EGCG (■) for 48 h. Relative number of viable cells was calculated as a ratio of number of viable cells treated with catechins to the number of cells cultured under the same conditions without catechin treatment and indicated as a percentage. For estimation of the synergistic effect of EC combined with EGCG, expected value for relative number of cells (▲) was calculated by adding the reduction from 100% in the relative number of the cells treated with EC (■) to that of the cells treated with 100 μM EGCG alone (the reduction in viable cells from 100% of EGCG-treated cells where the EC concentration is zero). The difference between the relative number of cells treated with EGCG and EC (◆) and the expected value (▲) indicates the synergistic effect of EC combined with EGCG on growth inhibition.
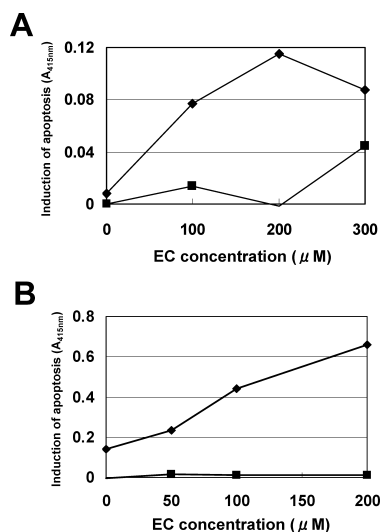
A

B

Fig. 6. Synergistic Induction of Apoptosis with EGCG and EC in KATO-III Cells and MKN-45 Cells

KATO-III cells (panel A) and MKN-45 cells (panel B) were treated with EC at the indicated concentrations with 100 μM EGCG (◆) or without EGCG (■) for 48 h. Induction of apoptosis was measured with an enzyme-linked assay for apoptosis.

Table 1. Synergistic Effect of EC Combined with Other Catechins on Growth Inhibition and Induction of Apoptosis in MKN-45 Cells

	Synergistic effect of EC (200 μM) combined with other catechins	
	Growth inhibition	Induction of apoptosis
EGCG (100 μM)	−	+++
EGC (100 μM)	+	+++
ECG (100 μM)	−	−

Synergistic effect of EC was indicated by the enhancing effect of EC on the growth inhibition or induction of apoptosis induced with EGCG, EGC or ECG as follows: −; no effect, + enhanced less than 1.5-fold, ++; 1.5 to 2-fold, +++; more than 2-fold.
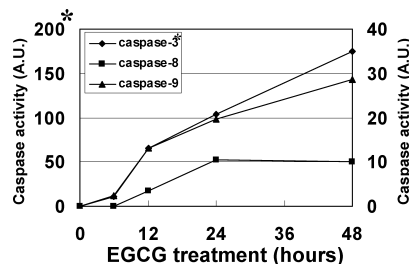

Fig. 7. Activation of Caspases by EGCG Treatment in MKN-45 Cells Measured by Enzyme Assay

Cell lysates were prepared from MKN-45 cells treated with EGCG and the activity of caspase-3 (◆), caspase-8 (■), and caspase-9 (▲) was measured with a Caspase/FLICE fluorometric protease assay kit. The fluorescent intensity of each reaction mixture in a 96-well microplate under UV illuminator (365 nm) was measured and indicated in arbitrary units. The activity of caspase-3 is indicated by the left ordinate (∗) and the activity of caspase-8, and -9 is indicated by the right ordinate.

duced by EGCG, EGC, and ECG.

Activation of Caspases-3, -8 and -9 in the Apoptosis Induced by EGCG Treatment　To determine the signaling pathway for the induction of apoptosis in the cells treated with EGCG, we examined whether specific caspases were involved. To determine the activity of caspases induced by EGCG treatment, we used a Fluorometric Protease Assay Kit with substrates specific for each caspase. Caspase-3 is commonly activated during apoptosis. In contrast, caspase-8 is activated in a pathway triggered by the death receptor, whereas caspase-9 is activated in a pathway involving physiological changes in mitochondria. As shown in Fig. 7, EGCG induced a significant increase in the activity of caspase-3 in MKN-45 cells as well as an increase in that of caspase-8 and caspase-9; however, the induction was weaker for caspase-8 than the other two caspases. We confirmed these results by Western blotting with antibodies against each caspase (Fig. 8). The results of Western blotting revealed that activation of caspases-3 and -9 was clearly induced by EGCG treatment; however, a significant activation of caspase-8 was observed in untreated MKN-45 cells and almost no change in the activation of caspase-8 was observed following the EGCG treatment.

Effect of Catalase Treatment on the Synergistic Apoptosis Induction by EC and EGCG Treatment　A large synergistic effect on the induction of apoptosis was observed when MKN-45 cells were treated with EC and other catechins (Table 1). To elucidate the mechanism of the synergistic effect of EC on the induction of apoptosis by EGCG treatment, we examined the influence of catalase treatment on the synergism. MKN-45 cells were treated with 100 μM EGCG

cally both in KATO-III cells and in MKN-45 cells (Fig. 6). Then, we examined the synergistic effect of EC in combination with other catechins on growth inhibition and induction of apoptosis in MKN-45 cells. The results, summarized in Table 1, suggest that EC has a significant synergistic effect on the induction of apoptosis in combination with 100 μM of EGCG or EGC, but little or no effect on growth inhibition in-
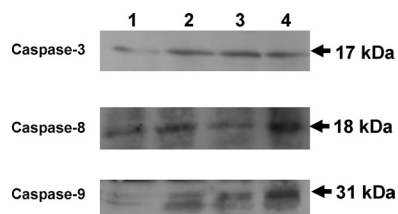
Fig. 8.   Activation of Caspases-3, -8, and -9 by EGCG Treatment Detected by Western Blotting

Cell lysates were prepared from MKN-45 cells cultured in complete media without EGCG (lane 1) or treated with 200 μM EGCG for 6 h (lane 2), 12 h (lane 3), and 24 h (lane 4) and activated caspases were detected by Western blotting. Arrows on the right side indicate bands due to activated caspases. Molecular weights of detected bands are indicated on the right side of the arrows. The 17 kDa and 18 kDa bands correspond to a large subunit of active form of caspase-3 and caspase-8, respectively. The 31 kDa band corresponds to intermediate products (the prodomain plus large subunit) during the activation of caspase.
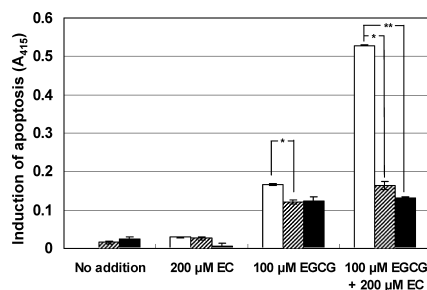

Fig. 9.   Effects of Catalase on Induction of Apoptosis by EGCG and EC Treatment in MKN-45 Cells

MKN-45 cells were treated without or with 200 μM EC and/or 100 μM EGCG. Catalase was added to the media 30 min before the addition of EC and/or EGCG at a final concentration of 0 U/ml (open bar), 50 U/ml (hatched bar) or 100 U/ml (closed bar). After incubation for 24 h, the induction of apoptosis was evaluated. Error bars indicate the standard deviation calculated from three independent experiments. Asterisks indicate significance (∗ for $p < 0.05$ and ∗∗ for $p < 0.01$) of difference between indicated data using a t test.

and/or 200 μM EC in the presence or absence of catalase. As shown in Fig. 9, catalase treatment slightly suppressed the apoptosis induced with EGCG alone. On the other hand, catalase treatment specifically canceled the synergistic effect of EC on the induction of apoptosis and the level of apoptosis induced by the treatment with EGCG and EC decreased to that induced with EGCG alone (Fig. 9).

DISCUSSION

The present study demonstrates that tea catechins possess activities for growth inhibition and induction of apoptosis in MKN-1, MKN-45, MKN-74, and KATO-III cells established from gastric carcinoma. As shown in Figs. 1 and 2, the IC$_{50}$ of four catechins contained in green tea for cell growth differed among the cell lines: MKN-45 cells were most susceptible to catechin treatment and the IC$_{50}$ of EGCG, EGC and ECG was about 40 μM. EC and C had almost no effect on the growth of MKN-45 cells. On the other hand, for KATO-III cells, which were the most insensitive to catechin treatment, the IC$_{50}$ for cell growth inhibition of EGCG, EGC and ECG was in the range of 80—150 μM, which is consistent with results reported previously.[12] Catechins induced apoptosis more effectively in MKN-45 cells than in KATO-III cells. As shown in Fig. 4, KATO-III cells required a 5- to 10-fold higher concentration of EGCG than did MKN-45 cells for apoptosis to be induced to the same extent. Among four cate-

chins contained in green tea, EGCG was most effective in inducing apoptosis and EGC had only weak activity for inducing apoptosis. It is noteworthy that ECG as well as EC and C had almost no effect on apoptosis in MKN-45 cells or KATO-III cells. The different activity profiles of the four catechins, EGCG, ECG, EGC and EC, for growth inhibition and induction of apoptosis suggested that these two activities are mediated by different mechanisms. On the other hand, MKN-45 cells are more sensitive to EGCG treatment than KATO-III cells in terms of both inhibition of growth and induction of apoptosis, suggesting that a common process underlies the inhibition and induction.

Different mechanisms for growth inhibition and induction of apoptosis were suggested from the results obtained in the analysis of the synergistic effect of EC and other catechins. As summarized in Table 1, EC and other catechins had a significant synergistic effect on the induction of apoptosis, but not on growth inhibition. These results suggested that the molecular mechanism for the induction of apoptosis by catechin differs from that for the inhibition of growth by the treatment.

To determine the pathway for the induction of apoptosis by EGCG treatment, we examined the activation of caspases during the induction process. In MKN-45 cells, a significant activation of caspase-3 and caspase-9 was demonstrated both by enzymatic assay and by the detection of activated fragments of caspases (Figs. 7, 8). In contrast, the activation of caspase-8 was lower than that of the other two caspases. These results suggested that both Fas and mitochondrial pathways are utilized for the induction of apoptosis; however, the contribution of the pathway including the activation of caspase-9 is major in the induction of apoptosis of MKN-45 cells on treatment with EGCG.

Then, we examined the mechanisms of the synergistic effect of EC and EGCG on the induction of apoptosis. Catalase treatment had little effect on the induction of apoptosis by EGCG treatment without EC, however, it specifically canceled the synergistic effect of EC on the induction of apoptosis by EGCG. These results suggested that the synergistic effect was mediated by the extracellular H_2O_2 produced by the EC and EGCG treatments and that the mechanism for induction of apoptosis by the treatment with EGCG alone utilizes mediators other than extracellular H_2O_2.

Many potential mechanisms have been proposed for the growth inhibition or induction of apoptosis in cultured cells treated with EGCG. These mechanisms include antioxidant/pro-oxidant activity of EGCG,[13] inhibition of MAP kinase signaling[14] including inactivation of AP-1 and inhibitor κB,[15] inhibition of growth factor signaling,[16] or direct binding to FAS.[10] In the present study, we have clearly shown that extracellular production of H_2O_2 contributes to the synergistic effect of EC on the apoptosis induced by EGCG treatment. Recent studies have suggested that the induction of apoptosis by tea polyphenols may be related to their pro-oxidant activity, including the production of H_2O_2. For example, EGCG-induced apoptosis in H661 lung cancer cells was blocked when catalase is included in the medium.[2] In the present study, the mediator for the apoptosis in MKN-45 cells treated with EGCG alone was suggested to be different from extracellular H_2O_2, which contributed to the apoptosis in MKN-45 cells treated with a combination of EC and

EGCG. The fact that caspase-9 as well as caspase-3 was activated in the induction of apoptosis suggests that the mitochondrial pathway was involved in the process and an increase in intracellular oxidation and/or reactive oxygen species (ROS) may contribute to the apoptosis induced by EGCG alone. In human leukemic cells, EGCG is suggested to induce an intracellular increase in ROS and the oxidative event triggered by EGCG treatment was reported to induce apoptosis *via* activation of oxidation-triggered c-Jun N-terminal kinase and p38 MAP kinase. In this case, the activation of caspase-9 and caspase-3 was observed during the induction of apoptosis.[9]

We evaluated growth inhibition from the reduction in viable cells after the catechin treatment compared to the untreated cells cultured under the same condition. Taking into account that the doubling time of cultured cells used in the study is about 24 h, the value less than 25% of relative number of viable cells in Figs. 1, 2 and 5 suggests an actual reduction in viable cells during the catechin treatment. As shown in Figs. 1 and 2, MKN-45 cells treated with EGCG at higher concentration than 50 μM or KATO-III cells treated with EGCG at higher than 200 μM for 48 h showed an actual reduction in viable cells. As shown in Fig. 4, where EGCG treatment caused an actual reduction in viable cells, apoptosis was induced both in MKN-45 cells and KATO-III cells. Furthermore, a large induction of apoptosis was observed in MKN-45 cells corresponding to an actual reduction in viable cells (Figs. 5, 6). These results suggest that growth inhibition with lower concentrations of EGCG without the actual reduction in viable cells results not from cell death but from the arrest of cell division and that the actual reduction in viable cells observed in Figs. 1, 2 and 5 results from apoptosis induced by catechin treatment. Many studies suggested that catechin treatment induces cell cycle arrest and recent study reported that EGCG induced cell cycle arrest in G1 phase.[17]

The concentration needed for the inhibition of cell growth or induction of apoptosis differed among the four cell lines examined although these cell lines were established from gastric carcinoma: the susceptibility of MKN-45 cells to EGCG treatment was 1.5—2-fold higher for growth inhibition and 15—10-fold higher for induction of apoptosis than that of KATO-III cells. A difference in the susceptibility of cell lines from gastric carcinoma was reported for SUN cell lines.[12] To evaluate the chemopreventing effect of green tea, it is noteworthy that relatively low concentrations of catechins can inhibit the growth of cells established from gastric carcinoma. Recent studies suggest that the systemic bioavailability of tea polyphenol is very low.[1] The concentration of catechins detected in plasma after the intake of green tea as a beverage is reported to be less than one micro mol.[18] A high concentration of catechins is only expected in the gastrointestinal tract including the gastric mucosa, where direct interaction with green tea occurs. The IC$_{50}$ of EGCG for the

growth inhibition of MKN-45 cells is about 40 μM and comparable to the intragastric concentration after consumption of green tea as a beverage.[1]

In summary, we examined the molecular mechanism of induction of apoptosis in gastric carcinoma cells treated with green tea catechins and showed that the mitochondrial pathway followed by the activation of caspases-3 and -9 is utilized as a major pathway. We also demonstrated that EC enhanced the induction of apoptosis caused by EGCG or ECG and that the enhancing effect is mediated by the extracellular production of H$_2$O$_2$. Although further studies are needed to establish that green tea as a beverage has chemopreventing effects, our results show that some kinds of gastric carcinoma are highly susceptible to catechin treatment and that catechins contained in green tea induce apoptosis in a synergistic manner, which suggest that the growth of susceptible types of gastric carcinoma cells is inhibited by the intake of green tea as a beverage.

REFERENCES

1) Yang C. S., Maliakal P., Meng X., *Annu. Rev. Pharmacol. Toxicol.*, **42**, 25—54 (2002).
2) Yang G.-Y., Liao J., Kim K., Yurkow E. J., Yang C. S., *Carcinogenesis*, **19**, 611—616 (1998).
3) Muramatsu K., Ogun I., Isemura M., Sugiyama K., Yamamoto-Maeda M., "Health Science of Tea," Japan Scientific Societies Press, Tokyo, 2002, pp. 52—64.
4) Roy M., Chakrabarty S., Sinha D., Bhattacharya R. K., Siddiqi M., *Mutation Res.*, 523—524, 33—41 (2003).
5) Lambert J. D., Yang C. S., *J. Nutr.*, **133**, 3262S—3267S (2003).
6) Stennicke H. R., Salvesen G. S., *Biochim. Biophys. Acta*, **1477**, 299—306 (2000).
7) Nagata S., *Exp. Cell Res.*, **256**, 12—18 (2000).
8) Ichijo H., *Oncogene*, **18**, 6087—6093 (1999).
9) Saeki K., Kobayashi N., Inazawa Y., Zhang H., Nishitoh H., Ichijo H., Saeki K., Isemura M., You A., *Biochem. J.*, **368**, 705—720 (2002).
10) Hayakawa S., Saeki K., Sazuka M., Suzuki Y., Shoji Y., Ohta T., Kaji K., You A., Isemura M., *Biochem. Biophys. Res. Commun.*, **285**, 1102—1106 (2001).
11) Suganuma M., Okabe S., Kai Y., Sueoka N., Sueoka E., Fujiki H., *Cancer Res.*, **59**, 44—47 (1999).
12) Okabe S., Ochiai Y., Aida M., Park K., Kim S.-J., Nomura T., Suganuma M., Fujiki H., *Jpn. J. Cancer Res.*, **90**, 733—739 (1999).
13) Yang G. Y., Liao J., Li C., Chung J., Yurkow E. J., Ho C. T., Yang C. S., *Carcinogenesis*, **21**, 2035—2039 (2000).
14) Dong Z., *Biofactors*, **12**, 17—28 (2000).
15) Yang F., Oz H. S., Barve S., de Villiers W. J., McClain C. J., Varilek G. W., *Mol. Pharmacol.*, **60**, 528—533 (2001).
16) Masuda M., Suzui M., Weinstein I. B., *Clin. Cancer Res.*, **7**, 4220—4229 (2001).
17) Gupta S., Hussan T., Mukhtar H., *Arch. Biochem. Biophys.*, **410**, 177—185 (2004).
18) Lee M. J., Maliakal P., Chen L., Meng X., Bondoc F. Y., Prabhu S., Lambert G., Mohr S., Yang C. S., *Cancer Epidemol. Biomarkers Prev.*, **11**, 1025—1032 (2002).