Liver International ISSN 1478-3223

BASIC STUDIES

Epigallocatechin-gallate modulates chemotherapy-induced apoptosis in human cholangiocarcinoma cells

Molly Lang[1], Roger Henson[1], Chiara Braconi[2] and Tushar Patel[1,2]

1 Scott and White Clinic, Texas A&M University Health Sciences Center, College of Medicine, Temple, TX, USA
2 Ohio State University, Columbus, OH, USA

Keywords
adjunct therapy – biliary tract cancers – chemotherapy – drug sensitivity – green tea

Abbreviations
EGC, Epigallocatechin; EGCG, epigallocatechin-gallate; 5-FU, 5-fluorouracil; GEM, gemcitabine; MMC, mitomycin C.

Correspondence
Tushar Patel, Ohio State University, 395 West 12th Avenue, 2nd floor, Columbus, OH 43210, USA
Tel: +614 366 4748
Fax: +614 293 8518
e-mail: tushar.patel@osumc.edu

Received 12 June 2008
Accepted 16 December 2008

DOI:10.1111/j.1478-3231.2009.01984.x

Abstract

Background: Green tea polyphenols are chemopreventive in several cancer models but their use as adjunctive therapeutic agents for cancer is unknown. *Aims:* Cholangiocarcinomas respond poorly to chemotherapeutic agents and our aims were to assess the utility of green tea polyphenols as adjuncts to chemotherapy for cholangiocarcinoma. *Materials and Methods:* We assessed the effect of purified green tea catechins on chemotherapy-induced apoptosis in KMCH, CC-LP-1 and Mz-ChA-1 human cholangiocarcinoma cells, and on chemosensitivity of Mz-ChA-1 cell xenografts in nude mice. *Results:* Epigallocatechin-gallate (EGCG), but not the structurally related catechin epigallocatechin, sensitized cells to apoptosis induced by gemcitabine (GEM), mitomycin C or 5-fluorouracil *in vitro*. Mitochondrial membrane depolarization, cytosolic cytochrome *c* expression and apoptosis were increased in cells incubated with EGCG and GEM compared with either agent alone. Furthermore, EGCG decreased *in vivo* growth and increased the sensitivity to GEM of Mz-ChA-1 cell xenografts in nude mice. *Conclusions:* The green tea polyphenol EGCG sensitizes human cholangiocarcinoma cells to chemotherapy-induced apoptosis and warrants evaluation as an adjunct to chemotherapy for the treatment of human cholangiocarcinoma.

Malignant tumours arising from the epithelial lining of the biliary tract, or cholangiocarcinomas, are rare but of considerable importance given their increasing incidence worldwide (1–4). These cancers have a poor prognosis. In part, this is related to their late presentation, when curative surgery may not be possible. Unfortunately, the tumours are highly refractory to conventional chemotherapeutic agents and therefore improved treatments for this tumour are urgently needed.

Epidemiological studies showing an inverse association between increased green tea intake and relative risk for cancer suggest that green tea may be useful as a therapeutic agent for cancer (5, 6). Although their role as therapeutic agents for cancer remains undefined, green tea polyphenols have been shown to be chemopreventive for several different cancers. A green tea-derived compound, polyphenon E, is currently in phase I/II clinical trials for chemoprevention and treatment of several cancers. Moreover, green tea extracts are readily available and often touted and promoted for a variety of health reasons, including many liver diseases (7).

Green tea polyphenols account for 30% of the dry weight of green tea and consist of several diverse catechins, of which the most abundant is epigallocatechin-gallate (EGCG). EGCG has been extensively studied and has been reported to be chemopreventive for many different cancers such as liver, prostate, stomach, oesophagus, colon, pancreas, bladder, skin, lung and breast. In addition, EGCG has chemopreventive effects in carcinogenesis induced by ultraviolet light, chemical agents and genetic aberrations (8). These observations suggest that EGCG may be potentially useful for the prevention or treatment of cancer, but this remains to be formally established.

Because of the growing literature on the potential benefits of green tea extracts and their ready availability, it is likely that they may be used by patients diagnosed with cancers seeking to improve their survival. However, the impact of green tea constituents on the growth or treatment of cholangiocarcinoma is unknown. Although published studies suggest that green tea extracts may be useful as adjunct treatments, the contributions of individual green tea constituent catechins to these effects are unclear. Our aims were to assess the effect of green tea polyphenols on chemotherapeutic responses in human cholangiocarcinoma cells to provide a rational basis for their use as adjuncts in the treatment of this otherwise poorly responsive cancer. We sought to address the following questions: Are green tea catechins toxic to cholangiocarcinoma cells? Do different catechins have similar effects on cholangiocarcinoma cells? What is their effect on chemosensitivity? Can they induce or protect against chemotherapy-induced apoptosis?

Experimental procedures

Cells

KMCH-1 human malignant cholangiocytes were obtained as previously described and cultured in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum, 1% antibiotic/antimycotic solution and 1% sodium pyruvate (9).

CC-LP-1 cells were kindly provided by Patricia Whiteside (University of Pittsburgh, Pittsburgh, PA, USA) and were cultured exactly as KMCH-1 cells. Mz-ChA-1 cells, human malignant cholangiocytes, were kindly provided by Dr. J. G. Fitz (University of Colorado, Denver, CO, USA) and were cultured in CMRL 1066 medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% antibiotic/antimycotic solution.

IC50 determinations

Cells were plated (10 000/well) in 96-well plates (BD Biosciences, Rockville, MD, USA) and incubated at 37 °C with varying concentrations of drug ranging from 10^{-3} to 10^{-9} M. Cell viability was assessed after 72 h using the CellTiter 96 Aqueous assay (Promega Corp., Madison, WI, USA) and the IC50 was determined by curve fitting using the XLFIT software (IDBS, Guildford, Surrey, UK).

Apoptosis assay

Cells with morphological changes indicative of cell death by apoptosis were identified and quantitated by fluorescence microscopy after staining with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence was visualized using a Nikon Eclipse TE300 inverted fluorescence microscope (Nikon Corp., Tokyo, Japan). Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation.

Western blot analysis

Western blot analysis was performed as previously described (10). Cells were grown to 70–80% confluency and then lysed. Protein samples were separated on 4–12% gradient polyacrylamide gels (Invitrogen, San Diego, CA, USA) and electroblotted to a positively charged 0.45 μM nitrocellulose membrane (Millipore, Bedford, CA, USA). Membranes were then incubated sequentially with primary antibody, used at a 1:1000 dilution, and then with a secondary polyclonal goat anti-rabbit immunoglobulin peroxidase conjugate (Zymed, San Francisco, CA, USA) at a 1:2000 dilution. Membranes were washed and then visualized using an enhanced chemiluminescence kit (LumiGLO; Cell Signaling, Beverly, MA, USA) and quantified by densitometry using a CCD camera-based image analyser (ChemiImager 4000; Alpha Innotech, San Leandro, CA, USA).

Analysis of drug interactions

Quantfication of the interactions between EGCG and gemcitabine (GEM) was performed as described previously (11). Cytotoxicity was assessed using a cell viability assay (CellTiter 96AQueous; Promega Corp) and the concentration at which 50% toxicity occurred (LD50) was determined. A median-effect analysis was performed and a combination index (CI) was derived based on the multiple drug effect equation of Chou and Talalay (12) and using the calcusyn program (Biosoft, Ferguson, MO, USA). A CI of < 1, 1 and > 1 define the interactions between two agents as synergistic, additive or antagonistic respectively.

Mitochondrial membrane potential (Δy_m) analysis

Alterations in Δy_m were analysed using the mitochondrial membrane potential (MMP)-sensitive cationic dye JC-1 (Cell Technology, Mountain View, CA, USA) and using the manufacturer's protocol. Cells were incubated with the agents at the indicated conditions, then were harvested and washed with phosphate-buffered saline (PBS). Cells (1×10^6) were then incubated with JC-1 (10 μg/ml) at 37 °C for 15 min in the dark. Stained cells were then washed with PBS and analysed using a fluorescence microplate reader to measure red fluorescence (excitation 530 nm, emission 620 nm) and green fluorescence (excitation 485 nm, emission 530 nm). JC-1 accumulates in mitochondria, whereupon J-aggregates form that are red fluorescent. Loss of the MMP is accompanied by a loss of mitochondrial accumulation and an increase in green fluorescence because of the monomeric cytoplasmic form of JC-1. A reduction in red:green fluorescence thus indicates loss of Δy_m and mitochondrial membrane depolarization.

Immunocytochemistry

Immunocytochemistry was performed as outlined in the SelectFX Alexa Fluor 488 Cytochrome *c* Apoptosis Detection Kit (Molecular Probes, Eugene, OR, USA). Anti-cytochrome *c* primary antibody was used at a dilution of 1:1000 and applied to samples for 2 h at room temperature. Alexa Fluor 488 goat anti-mouse secondary antibody was used at a dilution of 1:1000 and applied to samples for 30 min at room temperature. Slides were mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes). Imaging was performed using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a CCD camera. Fluorescence intensity in cytoplasmic regions was quantified in at least 10 cells/treatment group using the METAMORPH 6.2 (Universal Imaging Corp., Downingtown, PA, USA).

Transient transfections

The Bcl-2 expression plasmid was kindly provided by Dr Junying Yuan (Boston, MA, USA). Mz-ChA-1 cells (3×10^5) were plated in a 35 mm dish in culture media for 24 h before transient transfection with control or Bcl-2 expression plasmid using TransIT-LT1 (Mirus Corp., Madison, WI, USA) as described previously (13).

In vivo chemosensitivity

Studies were approved by the Institutional Animal Care and Use Committee. All animals received humane care according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male athymic nu/nu mice, 8 weeks old, were obtained from Harlan Teklad (Indianapolis, IN, USA). The mice were housed three per cage and fed *ad libitum*. Fluorescent light was controlled to provide alternate light and dark cycles of 12 h each and temperature and humidity were controlled. Mz-ChA-1 cells (5×10^6 viable cells suspended in 250 μL of extracellular matrix gel) were injected subcutaneously into the right and left flanks as described previously (14). The size of the resulting tumours was estimated three times per week by triaxial tumour measurements and tumour volume was estimated using the following equation: Tumour volume $(mm^3) = 4/3\ \pi$ [length (mm) × width (mm) × height (mm)]^3. Once xenografts had formed in all mice, mice were randomly assigned to one of four groups. Group A received intraperitoneal (i.p.) injections of saline daily for 10 days. Group B received i.p. injections of EGCG (20 mg/kg) daily for 10 days. The dose of EGCG used per body weight is similar to that used in human clinical studies of EGCG. Group C received i.p.

injections of GEM (120 mg/kg) on days 1, 4 and 7 only. Group D received i.p. injections of EGCG (20 mg/kg) daily for 10 days in addition to i.p. injections of GEM (120 mg/kg) 3 h following EGCG injections on days 1, 4 and 7 only.

Cell proliferation assay

Cell proliferation was measured using the CellTiter 96 AQueous assay kit (Promega Corp.). Mz-ChA-1 cells were plated (10 000 cells/well) in 96-well plates (BD Biosciences) and incubated at 37 °C and cell proliferation was assessed after 72 h as described previously (13). The study was repeated four times, each with five replicates.

Statistical analysis

Data are expressed as the mean ± 95% confidence intervals from at least three separate experiments performed in quadruplicate, unless otherwise noted. The differences between two groups were analysed using a two-sided Student's *t*-test. Statistical significance was considered as $P < 0.05$. Statistical analyses were performed with the GRAPHPAD IN-STAT statistical software program (Graph Pad, San Diego, CA, USA).

Materials

CMRL 1066 and DMEM media, L-glutamine, sodium pyruvate, fetal bovine serum and antibiotic–antimycotic solutions were from Gibco BRL (Grand Island, NY, USA). GEM was obtained from Eli Lilly (Indianapolis, IN, USA). Antibody to the 67 kDa laminin receptor was obtained from Abcam (Cambridge, MA, USA) and antibody to caspase-8 and caspase-9 were obtained from New England Biolabs (Ipswich, MA, USA). 5-fluorouracil (5FU), camptothecin, EGCG, epigallocatechin (EGC) and all other catechins were obtained from Sigma Aldrich (St Louis, MO, USA). Mitomycin C (MMC) and DAPI were obtained from Calbiochem (La Jolla, CA, USA). All other reagents were of analytical grade from the usual commercial sources.

Results

Chemotherapeutic agents induce apoptosis in human cholangiocarcinoma cell lines

We began by assessing the effect of chemotherapeutic agents on apoptosis in human cholangiocarcinoma cell lines. Firstly, we evaluated the effect of the chemotherapeutic agents GEM, 5FU and MMC on apoptosis in Mz-ChA-1 cells derived from metastatic gall bladder cancer. All agents induced apoptosis, with the effect being greatest for GEM (Fig. 1A). These *in vitro* data parallel the clinical experience of these agents for the treatment of biliary tract cancers in which the best survival rates for therapy with a single agent has been observed with GEM (15, 16). Next, we assessed the effect of GEM on other cell types, CC-LP-1 and TFK-1 derived from an intrahepatic and extrahepatic biliary tract respectively. GEM induced apoptosis to a similar extent in all cell lines (Fig. 1B).

Plant-derived catechins have diverse effects on apoptosis

In order to assess whether the addition of green tea-derived polyphenols could modulate chemosensitivity, we initially assessed the effect of selected purified catechins and the corresponding catechin-gallates on apoptosis (Fig. 2). The most abundant catechins, EGC and EGCG, did not significantly

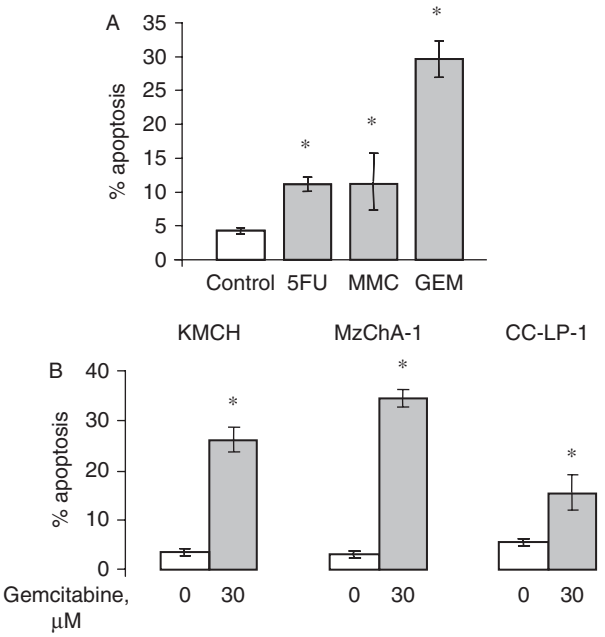

Fig. 1. Chemotherapeutic agents induce apoptosis in human cholangiocarcinoma cell lines. (A) The response of Mz-ChA-1 cells to diverse chemotherapeutic agents was assessed. Using a viable cell assay, the IC50 for MzChA-1 cells at 72 h for 5-fluorouracil, mitomycin C and gemcitabine (GEM) was determined to be 42, 51 and 28 μM respectively. Cells were incubated at their IC50 concentrations and the extent of apoptosis was assessed after 24 h. All three chemotherapeutic agents increased apoptosis in Mz-ChA-1 cells *in vitro*. The data represent mean ± 95% confidence intervals of three experiments, $^*P < 0.05$ compared with controls. (B) KMCH, Mz-ChA-1 and CC-LP-1 human cholangiocarcinoma cell lines were incubated in the presence or absence of GEM 30 μM. Apoptosis was assessed after 48 h. GEM induced apoptosis in all three cell lines. The data represent mean ± 95% confidence intervals of three separate studies. $^*P < 0.05$ compared with untreated controls.

induce apoptosis in Mz-ChA-1 cells compared with controls. Furthermore, no difference was observed between these two compounds. In contrast, other compounds such as catechin-gallate, gallocatechin-gallate and epicatechin-gallate induced apoptosis to variable extents. Surprisingly, despite their structural similarities, significantly greater apoptosis was observed in the presence of a gallate compared with the corresponding catechin. These findings suggest that the presence of a gallate moiety can promote apoptosis. We chose EGCG for further investigation because it is the most abundant catechin in green tea, is readily available in purified form and has been extensively investigated as a chemopreventive agent. Moreover, EGCG was not cytotoxic to non-malignant human cholangiocytes at the concentrations studied (data not shown). The fact that EGC showed very comparable apoptosis results to EGCG prompted its use as a structurally related negative control for our studies.

Epigallocatechin-gallate increases chemotherapy-induced apoptosis

Binding to the 67 kDa laminin receptor has been shown to mediate the anticancer effects of EGCG (17). We began by assessing the expression of this protein in human

Liver International (2009)

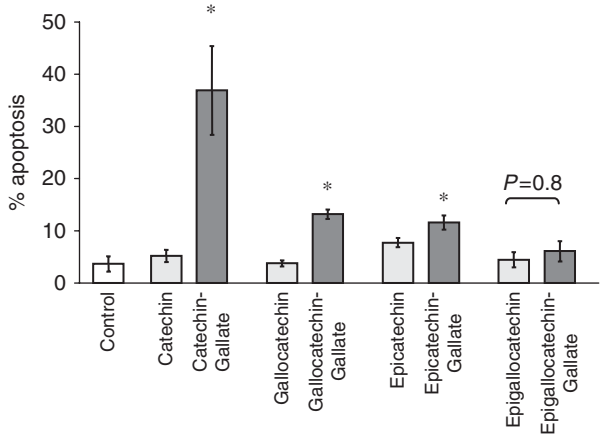

Fig. 2. Plant-derived catechins have diverse effects on apoptosis. The effect of pre-incubation with several plant-derived catechins on gemcitabine (GEM)-induced apoptosis was assessed in Mz-ChA-1 cells. Cells were pre-incubated with 1 μM of each catechin for 1 h. The media was then replaced with media containing 30 μM GEM. After 24 h, the number of cells undergoing apoptosis was counted after staining with DAPI. The data represent mean ± 95% confidence intervals from three to four separate studies.

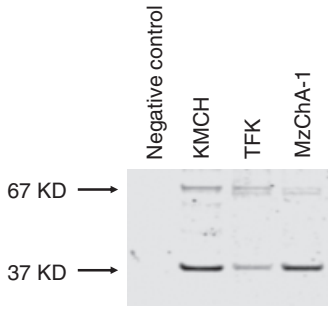

Fig. 3. The 67 kDa laminin receptor is expressed in human malignant cholangiocytes. Lysates were obtained from human cholangiocarcinoma cells grown to near confluency. Fifty micrograms of protein was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotted using an antibody that recognizes both the 67 kDa laminin receptor and its 37 kDa precursor. Expression of the 67 kDa laminin receptor was detected in all cell lines.

cholangiocarcinoma cell lines by immunoblot analysis. The laminin receptor was expressed on all three cell lines (Fig. 3). Next, we assessed the effect of pre-incubation with either EGCG or EGC on chemotherapy-induced apoptosis in Mz-ChA-1 cells. Although EGCG by itself did not appreciably enhance apoptosis, EGCG significantly increased chemotherapy-induced apoptosis (Fig. 4). This effect was specific for EGCG because pre-incubation with EGC did not significantly increase chemotherapy-induced apoptosis. Similar results were observed for all three agents studied – GEM, 5FU and MMC. Next, we assessed whether these effects also occurred in other human cholangiocarcinoma cell lines. KMCH, Mz-ChA-1 and CC-LP-1 cells were pre-incubated with catechin alone, GEM alone or with the combination of catechin and GEM. Similar to

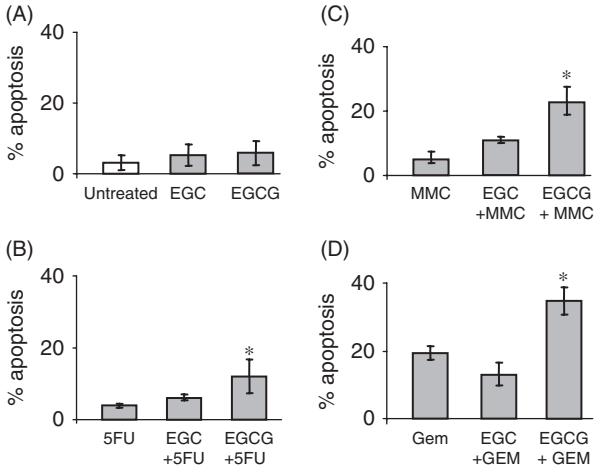

Fig. 4. Epigallocatechin-gallate (EGCG), but not epigallocatechin (EGC), increases chemotherapy-induced apoptosis. MzChA-1 cells were pre-incubated with the plant catechins EGC (1 μM), EGCG (1 μM) or diluent controls for 1 h before treatment with (A) diluent (controls), (B) 40 μM 5-fluorouracil, (C) 50 μM mitomycin C or (D) 30 μM gemcitabine. Apoptosis was assessed after 24 h. For all the agents, EGCG, but not EGC, increased chemotherapy-induced apoptosis. The data represent mean ± 95% confidence intervals from four separate studies. $^{*}P < 0.05$ compared with chemotherapeutic drug alone.

our observations with the Mz-ChA-1 cells, EGCG, but not EGC increased chemotherapy-induced apoptosis (Fig. 5). Taken together, these data suggest that EGCG may act on a common pathway involved in modulating apoptosis rather than on a drug-specific pathway.

We next examined the interactions between EGCG and GEM on drug-induced cytotoxicity in Mz-ChA-1 cells. The nature of the interaction was quantified using the median effects analysis to derive a CI. Combinations of EGCG and GEM were synergistic with a CI of 0.32 ± 0.19 for GEM at an effective dose for 50% cytotoxicity (ED50). Similar results were observed with EGCG, with a CI of 0.69 ± 0.17. Thus, EGCG has a synergistic effect to GEM in malignant cholangiocytes *in vitro*.

Epigallocatechin-gallate increases gemcitabine-induced loss of mitochondrial membrane potential

Our focus now turned to understanding the mechanism by which EGCG could enhance chemotherapy-induced apoptosis. Several non-mutually exclusive pathways leading to apoptosis have been described. We postulated that EGCG modulated the mitochondrial pathway of apoptosis, which involves mitochondrial membrane depolarization in response to an apoptosis-inducing stimulus, with subsequent release of factors such as cytochrome *c* that are capable of activating downstream effector caspases and inducing apoptosis. To test this hypothesis, we assessed the effect of incubation with EGCG, or EGC as a control, on loss of MMP in response to GEM. MMP was assessed as the ratio of red to green fluorescence resulting from the addition of a JC-1 dye to the cultures. A significant loss of the MMP was seen only in cells treated with the EGCG/GEM combination (Fig. 6). Next, we assessed the effect of EGCG and GEM on cytochrome *c* release. Immunocytochemistry for

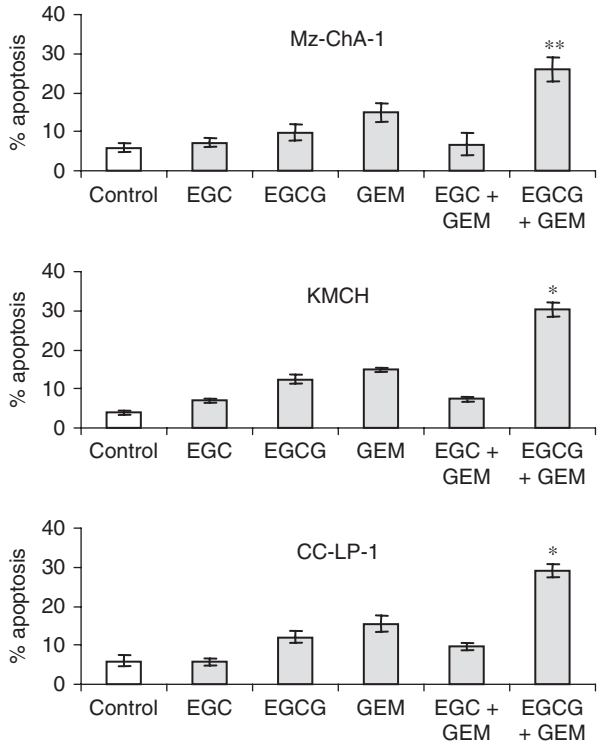

Fig. 5. Epigallocatechin-gallate (EGCG) increases gemcitabine (GEM)-induced apoptosis in cholangiocarcinoma cell lines. KMCH, MzChA-1 or CC-LP-1 cells were pre-incubated with the plant catechins epigallocatechin (EGC) (1 μM), EGCG (1 μM) or diluent (control) for 1 h before treatment with 30 μM GEM or diluent. Apoptosis was assessed after 24 h. EGCG, but not EGC, increased GEM-induced apoptosis in all three human cholangiocarcinoma cell lines. The data represent mean ± 95% confidence intervals from three studies. $^{*}P < 0.01$, $^{**}P < 0.05$ compared with GEM alone.

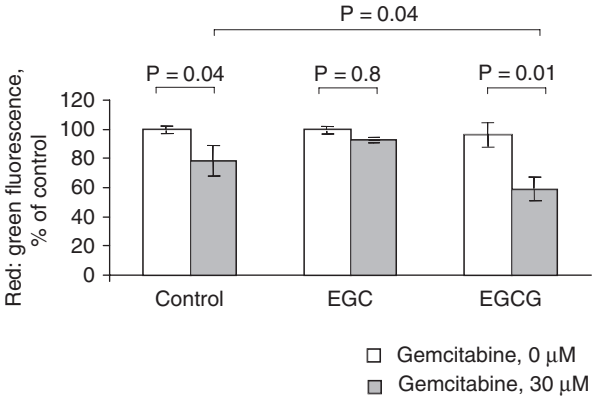

Fig. 6. Epigallocatechin-gallate (EGCG) increases gemcitabine (GEM)-induced loss of mitochondrial membrane potential (MMP). Mz-ChA-1 cells were pre-incubated with diluent controls, 1 μM epigallocatechin or 1 μM EGCG for 1 h before incubation with either diluent alone or 30 μM GEM for 24 h. 1×10^5 cells were then labelled with 10 μg/ml JC-1 for 30 min and the fluorescence was measured. The loss of MMP was assessed as the change in ratio of red to green fluorescence with loss of MMP, causing an increase in green fluorescence. EGCG decreases the red:green fluorescence ratio during incubation with GEM consistent with enhanced loss of MMP.

cytochrome *c* was performed and cytoplasmic cytochrome *c* was quantified (Fig. 7). An increase in cytoplasmic cytochrome *c* was observed only in cells treated with the combination of EGCG and GEM. Loss of cytochrome *c* can be inhibited by overexpression of the anti-apoptotic protein Bcl-2, which can modulate mitochondrial membrane permeabilization (18). In Mz-ChA-1 cells transiently transfected to overexpress Bcl-2, there was an inhibition of EGCG/GEM cytotoxicity by 40.1 ± 9.7%. These findings indicate that EGCG can act on mitochondria to enhance cytochrome *c* release and thereby modulate GEM-induced apoptosis. Upon release from the mitochondria, cytochrome *c* forms a complex with procaspase-9 and Apaf 1 resulting in activation of procaspase-9 by cleavage to an active 35 or 17 kDa fragment. Consistent with this effect, we observed a predominant increase in the expression of the 17 kDa active caspase-9 cleavage product but not the 18 kDa active caspase-8 cleavage product by immunoblot analysis (Fig. 8).

Epigallocatechin-gallate decreases cholangiocarcinoma xenograft growth and enhances the sensitivity to gemcitabine

In order to evaluate these effects *in vivo*, we studied the effect of administration of EGCG and GEM on the growth of Mz-ChA-1 cell xenografts in athymic nude mice. Once xenografts had grown to a predetermined size, the animals received a course of saline (control group) or treatment with EGCG alone, GEM alone or the combination of EGCG and GEM for 10 days. When compared with the control group, there was a significant decrease in tumour growth in each group over a 2-week period (Fig. 9). The combination of EGCG and GEM decreased tumour growth to the greatest degree thus corroborating our results found *in vitro* and supporting the hypothesis that EGCG enhances the sensitivity of Mz-ChA-1 cells to GEM treatment. Of note, treatment with EGCG alone also showed a significant reduction in tumour growth even though EGCG did not induce apoptosis or increase cytotoxicity in our *in vitro* studies. Incubation with 10 μM EGCG decreased Mz-ChA-1 cell proliferation *in vitro* by 21 ± 3% after 72 h. Thus, it is likely that EGCG has antitumour effects through other mechanisms and provides a stronger rationale for the use of EGCG as an adjunct in combination with GEM for the treatment of cholangiocarcinoma.

Discussion

In this study, we have demonstrated an effect of the green tea polyphenol EGCG on enhancing apoptosis induced by chemotherapeutic agents in malignant human cholangiocarcinoma cells. Induction of apoptosis is a mechanism by which chemotherapeutic agents may exert an antitumoral effect. EGCG enhanced the effect of GEM and, moreover, decreased the growth of cholangiocarcinoma xenografts *in vivo*. After oral administration, ECGC in plasma is mostly in the free form. The major route of excretion is biliary. Bioavailability is poor and oral administration of EGCG in rats resulted in recovery of 3.28% of the dose in the bile. However, of this, 80.1% was free EGCG and the remainder represented various methylated metabolites (19–21). Although the biological activity of the methylated metabolites is unknown, cholangiocarcinoma can

Liver International (2009)

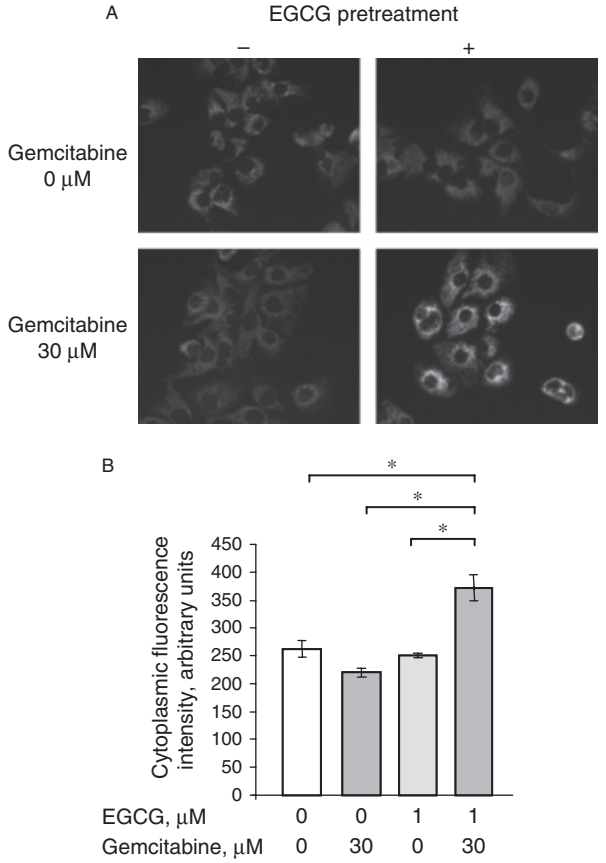

Fig. 7. Epigallocatechin-gallate (EGCG)+gemcitabine (GEM) increase cytoplasmic cytochrome *c* release. (A) Mz-ChA-1 cells were pre-incubated for 1 h with 1 μM EGCG or diluent control before treatment with either 0 or 30 μM GEM for 24 h. Immunocytochemistry was performed for cytochrome *c* and images were obtained by fluorescence microscopy. (B) The fluorescence intensity was measured in cellular cytoplasmic regions in all cells in a randomly selected field using the METAMORPH 6.2 image analysis software. The data represent mean ± 95% confidence intervals from 11 to 13 cells in each group. $^*P < 0.01$.

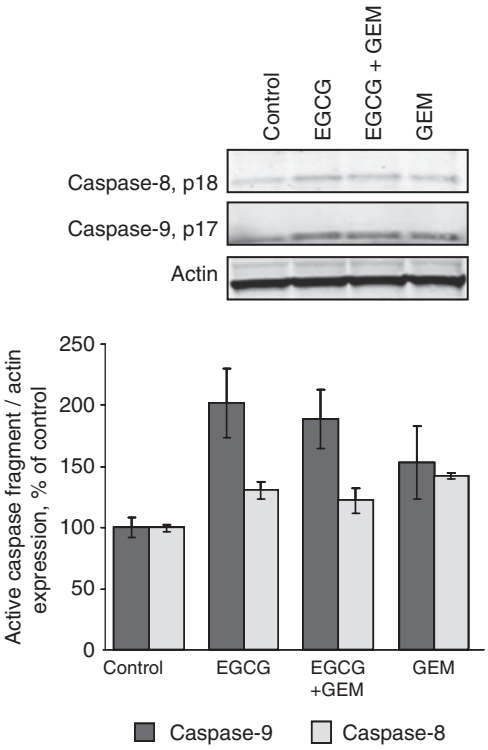

Fig. 8. Epigallocatechin-gallate (EGCG) enhances cleavage of caspase 9. MzChA-1 cells were incubated with 1 μM EGCG, 30 μM gemcitabine (GEM) or the combination of 1 μM EGCG and 30 μM GEM in serum-free medium. After 24 h, cell lysates were obtained and evaluated for the expression of caspase-8 and caspase-9 by Western blotting. Representative blots of the cleaved form of the caspases are shown along with quantitative data representing the average and the standard error of expression of active caspase cleavage products relative to the expression in control cells from four separate determinations.

be exposed to EGCG in both bile and blood. These studies provide a strong rationale for proceeding to patient-based studies to evaluate and define the therapeutic utility of EGCG and, in particular, its use as an adjunct to GEM and other chemotherapeutic agents for the treatment of cholangiocarcinoma.

Many chemotherapeutic drugs induce apoptosis via activation of the intrinsic mitochondrial pathway, which is negatively regulated by the apoptosis regulators Bcl-2 and Bcl-X_L (22). Ample evidence exists supporting a critical role for these two anti-apoptotic molecules in mediating broad-spectrum resistance to chemotherapy. The mechanism of chemosensitization by EGCG involves modulation of apoptosis via mitochondrial membrane depolarization and release of cytochrome *c*. The involvement of mitochondria is additionally supported by observations that EGCG can bind to and strongly inhibit Bcl-X_L and that the effects of EGCG are decreased by overexpression of Bcl-2. Moreover, EGCG has recently been shown to directly induce apoptosis in some cancer cell lines by modulating mitochondrial release of cytochrome *c*. In contrast to these studies, we did not observe a significant pro-apoptotic effect of EGCG alone in any of the three cholangiocarcinoma cell lines studied. Cholangiocarcinomas are highly resistant to most chemotherapeutic agents probably because of the presence of constitutively active intrinsic survival mechanisms. The presence of such mechanisms may attenuate direct induction of apoptosis by EGCG in cholangiocarcinoma cells. Nevertheless, EGCG enhances apoptosis in response to chemotherapeutic stress, suggesting that EGCG may sensitize cells and bring them closer to the tipping point at which intrinsic survival mechanisms cannot afford protection to cytotoxic agents. Although EGCG acts as an antioxidant by inhibiting lipid peroxidation or trapping reactive oxygen species, EGCG can also enhance oxidative stress by the generation of hydrogen peroxides (16). We speculate that the relative amounts of endogenous antioxidants may contribute to direct damage to mitochondrial membranes and activation of cellular stress mechanisms by EGCG and to the varying ability of EGCG to directly induce apoptosis. Elucidation of the precise mechanisms by which EGCG modulates mitochondrial membrane depolarization and cytochrome *c* release may provide new insights into mechanisms, by which tumour chemosensitivity may be enhanced.

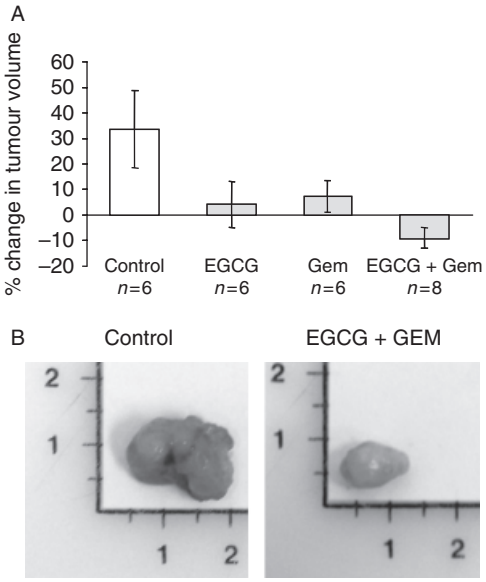

Fig. 9. Epigallocatechin-gallate (EGCG) decreases cholangiocarcinoma xenograft growth and enhances the sensitivity to gemcitabine (GEM). (A) Nude mice with tumour cell xenografts were treated with (a) intraperitoneal (i.p.) injections of saline daily for 10 days (three animals), (b) i.p.injections of EGCG (20 mg/kg) daily for 10 days (three animals) (c) i.p injections of GEM (120 mg/kg) every third day for three doses (three animals) or (d) i.p. injections of EGCG (20 mg/kg) daily for 10 days in addition to i.p. injections of GEM (120 mg/kg) 3 h following EGCG injections on days 1, 4 and 7 only. Tumour growth was assessed as percentage change in tumour volume compared with initial volume for each tumour over a 2-week period. Data represent mean ± 95% confidence intervals from six to eight tumours for each group. $^{*}P < 0.05$ compared with controls, $^{**}P < 0.05$ compared with GEM alone. (B) Representative tumours from control or EGCG+GEM-treated tumours, excised 25 days after completion of treatment.

Epigallocatechin-gallate is a major component of green tea. Several green tea extracts are available over-the-counter, which contain mixtures of EGCG and other catechins. However, green tea catechins differed in their ability to induce apoptosis. For most agents, these differences reflected the presence or absence of a gallate moiety, with the catechin-gallates inducing a greater degree of apoptosis compared with the respective catechins alone. Gallic acid has been reported to induce apoptosis in several cancer cell lines by a variety of mechanisms, including generation of reactive oxygen species and modulation of calcium influx and activation of calmodulin (23). Thus, the differences in the ability of green tea catechins to induce apoptosis may reflect hydrolysis, with the generation of gallic acid. However, neither EGC nor EGCG induced apoptosis by themselves but differed in their chemosensitizing effect in cholangiocarcinoma cells. EGCG and EGC dramatically differ in their ability to bind to Bcl-X_L and the presence of the gallate group in EGCG enhances docking in the BH3 domain (24). Thus, the differences between EGC and EGCG are more likely to reflect true structure–function effects.

While we have focused on studies to evaluate the effect of EGCG and other green tea catechins on chemotherapy-induced apoptosis, there are other potential mechanisms by which these compounds can potentiate the effects of chemotherapeutic agents. EGCG can modulate the expression of the multidrug resistance proteins (25). Moreover, EGCG and its metabolites are substrates for drug efflux pumps and can compete with chemotherapeutic drugs (26, 27). By saturating these efflux mechanisms, the effect of chemotherapeutic drugs can be increased. Studies to explore the mechanisms by which EGCG can decrease tumour growth *in vivo* are also warranted. EGCG can affect several different biological pathways (28). The p38 MAPK signalling is aberrantly activated in cholangiocarcinoma and inhibition of p38 MAPK signalling decreases cholangiocarcinoma growth both *in vivo* and *in vitro* (29) However, EGCG has been shown to decrease activation of p38 MAPK in some but not all studies (30–32). EGCG has been shown to modulate growth-factor signalling pathways and, additionally, has effects on cell-cycle progression, angiogenesis and tumour cell invasion (28, 33–35). Furthermore, EGCG can inhibit DNA methyltransferase activity in protein extracts and in human cancer cell lines and reactivate methylation-silenced genes (36). Thus, there are multiple mechanisms by which EGCG may have an antitumour effect.

Epigallocatechin-gallate has been extensively studied and shown to be pharmacologically safe. However, cholestasis is a common feature of biliary tract malignancies associated with obstruction and the effects of cholestasis on toxicity are unknown. Recent case reports have alerted to the possibility of acute hepatitis in patients taking supplements containing EGCG or green tea extracts (37–39). Although EGCG cannot be definitely incriminated because of the presence of several other compounds in these preparations, these reports emphasize the need for monitoring for potential hepatotoxicity and for phase I studies with escalating doses of EGCG, to evaluate the safety and efficacy and to identify tolerable doses in patients with cholestasis. Chemotherapeutic regimens are limited by non-specific toxicity towards normal cells and tissues. The use of adjunct EGCG may allow lower doses of conventional chemotherapeutic agents to be used and hopefully improve outcomes for cholangiocarcinoma by reducing toxicity and improving effectiveness.

Acknowledgements

This study was supported by the Scott & White Hospital Foundation and Grant DK069370 from the National Institutes of Health. The technical assistance provided by Valorie Chiasson, Linda Brooks and Janna Mize-Berge and administrative assistance provided by Melinda Freed are gratefully acknowledged.

References

1. Khan SA, Taylor-Robinson SD, Toledano MB, *et al*. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol* 2002; **37**: 806–13.
2. Patel T. Cholangiocarcinoma. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 33–42.
3. Patel T. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 2001; **33**: 1353–7.
4. Tadlock L, Yamagiwa Y, Hawker J, Marienfeld C, Patel T. Transforming growth factor-{beta} inhibition of proteasomal activity. A potential mechanism of growth arrest. *Am J Physiol Cell Physiol* 2003; **285(2)**: 277–85.

5. Blot WJ, Chow WH, McLaughlin JK. Tea and cancer: a review of the epidemiological evidence. *Eur J Cancer Prev* 1996; **5**: 425–38.
6. Ji BT, Chow WH, Hsing AW, *et al*. Green tea consumption and the risk of pancreatic and colorectal cancers. *Int J Cancer* 1997; **70**: 255–8.
7. Jin X, Zheng RH, Li YM. Green tea consumption and liver disease: a systematic review. *Liver Int* 2008; **28**: 990–6.
8. Lambert JD, Yang CS. Mechanisms of cancer prevention by tea constituents. *J Nutr* 2003; **133**: 3262S–7S.
9. Park J, Tadlock L, Gores GJ, Patel T. Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. *Hepatology* 1999; **30**: 1128–33.
10. Taylor-Robinson SD, Toledano MB, Arora S, *et al*. Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968–1998. *Gut* 2001; **48**: 816–20.
11. Yamagiwa Y, Marienfeld C, Tadlock L, Patel T. Translational regulation by p38 mitogen-activated protein kinase signaling during human cholangiocarcinoma growth. *Hepatology* 2003; **38**: 158–66.
12. Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**: 27–55.
13. Tadlock L, Patel T. Involvement of p38 mitogen-activated protein kinase signaling in transformed growth of a cholangiocarcinoma cell line. *Hepatology* 2001; **33**: 43–51.
14. Marienfeld C, Tadlock L, Yamagiwa Y, Patel T. Inhibition of cholangiocarcinoma growth by tannic acid. *Hepatology* 2003; **37**: 1097–104.
15. Dingle BH, Rumble RB, Brouwers MC. The role of gemcitabine in the treatment of cholangiocarcinoma and gallbladder cancer: a systematic review. *Can J Gastroenterol* 2005; **19**: 711–6.
16. Park JS, Oh SY, Kim SH, *et al*. Single-agent gemcitabine in the treatment of advanced biliary tract cancers: a phase II study. *Jpn J Clin Oncol* 2005; **35**: 68–73.
17. Stoner GD, Casto BC. Chemoprevention by fruit phenolic compounds. In: Kelloff GJ, Hawk ET, Sigman CC, eds. *Cancer Chemoprevention: Promising Cancer Chemoprevention Agents*. Totowa, NJ: Humana Press, 2004; 419–35.
18. Breckenridge DG, Xue D. Regulation of mitochondrial membrane permeabilization by BCL-2 family proteins and caspases. *Curr Opin Cell Biol* 2004; **16**: 647–52.
19. Suganuma M, Okabe S, Oniyama M, *et al*. Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis* 1998; **19**: 1771–6.
20. Lambert JD, Lee MJ, Lu H, *et al*. Epigallocatechin-3-gallate is absorbed but extensively glucuronidated following oral administration to mice. *J Nutr* 2003; **133**: 4172–7.
21. Lambert JD, Yang CS. Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. *Mutat Res* 2003; **523–524–4**: 201–8.
22. Kaufmann SH, Vaux DL. Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. *Oncogene* 2003; **22**: 7414–30.
23. Sartippour MR, Shao ZM, Heber D, *et al*. Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *J Nutr* 2002; **132**: 2307–11.
24. Leone M, Zhai D, Sareth S, *et al*. Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins. *Cancer Res* 2003; **63**: 8118–21.
25. Mei Y, Qian F, Wei D, Liu J. Reversal of cancer multidrug resistance by green tea polyphenols. *J Pharm Pharmacol* 2004; **56**: 1307–14.
26. Hong J, Lambert JD, Lee SH, Sinko PJ, Yang CS. Involvement of multidrug resistance-associated proteins in regulating cellular levels of (-)-epigallocatechin-3-gallate and its methyl metabolites. *Biochem Biophys Res Commun* 2003; **310**: 222–7.
27. Hong J, Lu H, Meng X, *et al*. Stability, cellular uptake, biotransformation, and efflux of tea polyphenol (-)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. *Cancer Res* 2002; **62**: 7241–6.
28. Moyers SB, Kumar NB. Green tea polyphenols and cancer chemoprevention: multiple mechanisms and endpoints for phase II trials. *Nutr Rev* 2004; **62**: 204–11.
29. Tachibana H, Koga K, Fujimura Y, Yamada K. A receptor for green tea polyphenol EGCG. *Nat Struct Mol Biol* 2004; **11**: 380–1.
30. Andreadi CK, Howells LM, Atherfold PA, Manson MM. Involvement of Nrf2, p38, B-raf and NF-{kappa}B, but not PI3K, in induction of hemeoxygenase (HO-1) by dietary polyphenols. *Mol Pharmacol* 2006; **69(3)**: 1033–40.
31. Balasubramanian S, Efimova T, Eckert RL. Green tea polyphenol stimulates a Ras, MEKK1, MEK3, and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. *J Biol Chem* 2002; **277**: 1828–36.
32. Chen W, Dong Z, Valcic S, Timmermann BN, Bowden GT. Inhibition of ultraviolet B-induced c-fos gene expression and p38 mitogen-activated protein kinase activation by (-)-epigallocatechin gallate in a human keratinocyte cell line. *Mol Carcinog* 1999; **24**: 79–84.
33. Jung YD, Kim MS, Shin BA, *et al*. EGCG, a major component of green tea, inhibits tumour growth by inhibiting VEGF induction in human colon carcinoma cells. *Br J Cancer* 2001; **84**: 844–50.
34. Kazi A, Smith DM, Daniel K, *et al*. Potential molecular targets of tea polyphenols in human tumor cells: significance in cancer prevention. *In Vivo* 2002; **16**: 397–403.
35. Patel T. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer* 2002; **3**: 2–10.
36. Fang MZ, Wang Y, Ai N, *et al*. Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res* 2003; **63**: 7563–70.
37. Bonkovsky HL. Hepatotoxicity associated with supplements containing Chinese green tea (*Camellia sinensis*). *Ann Intern Med* 2006; **144**: 68–71.
38. Gloro R, Hourmand-Ollivier I, Mosquet B, *et al*. Fulminant hepatitis during self-medication with hydroalcoholic extract of green tea. *Eur J Gastroenterol Hepatol* 2005; **17**: 1135–7.
39. Jimenez-Saenz M, Martinez-Sanchez MD. Acute hepatitis associated with the use of green tea infusions. *J Hepatol* 2006; **44(3)**: 616–7.