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Antiproliferative activity and induction of apoptosis in human colon cancer cells treated *in vitro* with constituents of a product derived from *Pistacia lentiscus* L. var. *chia*

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Abstract

In this report, we demonstrate that a 50% ethanol extract of the plant-derived product, Chios mastic gum (CMG), contains compounds which inhibit proliferation and induce death of HCT116 human colon cancer cells *in vitro*. CMG-treatment induces cell arrest at G_1, detachment of the cells from the substrate, activation of pro-caspases-8, -9 and -3, and causes several morphological changes typical of apoptosis in cell organelles. These events, furthermore, are time- and dose-dependent, but p53- and p21-independent. Apoptosis induction by CMG is not inhibited in HCT116 cell clones expressing high levels of the anti-apoptotic protein, Bcl-2, or dominant-negative FADD, thereby indicating that CMG induces cell death via a yet-to-be identified pathway, unrelated to the death receptor- and mitochondrion-dependent pathways. The findings presented here suggest that CMG (a) induces an anoikis form of cell death in HCT116 colon cancer cells that includes events associated with caspase-dependent pathways; and (b) might be developed into a chemotherapeutic agent for the treatment of human colon and other cancers.

Keywords: Chios mastic gum; Colon cancer cells; Apoptosis; *Pistacia lentiscus* L. var. *chia*

Introduction

Dietary intake of phytochemicals has been associated with decreased risk of cancer and significant survivability of cancer patients (Ho et al., 2002; Setzer and Setzer, 2003; Weiss and Landauer, 2003). Several plant products contained in foods have exhibited activity against human colon cancer. Buckwheat protein (Brivida et al., 2002), resveratrol analogs (Wolter et al., 2001, 2002), linoleic acid conjugates (Kemp et al., 2003), and green tea extracts (Mueller-Klieser et al., 2002) have exhibited anticancer activity *in vitro* by targeting various molecular and cellular mechanisms, and juice or freeze-dried powder from Brussels sprouts significantly enhanced levels of apoptosis and reduced mitosis in the colonic crypts in an animal model (Smith et al., 2003).

In most cases, anticancer drug treatment results in the activation of the enzymes, caspases, which act effectively in the execution of various forms of cell death. The

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doi:10.1016/j.phymed.2006.03.009

death receptor-dependent apoptotic pathway is triggered at the cell surface and requires activation of caspase-8, whereas the mitochondrion-dependent pathway is initiated with release of mitochondrial cytochrome *c* into the cytoplasm and requires activation of caspase-9. Subsequently, caspase-8 or -9 can activate caspase-3, which in turn targets and degrades specific and vital cellular proteins, ultimately resulting in nuclear DNA degradation and apoptotic death of the cells (for reviews on caspases, see Krammer and Debatin, 2004; Strasser et al., 2001). There is accumulating evidence of existence of caspase-independent mechanisms of cell death executed by other proteases, however, thus leading to variant forms that may display some or no characteristics of the "classical" apoptotic pathways (Jäättelä, 2004; Lockshin and Zakeri, 2004).

The tumor suppressor protein, p53, functions as a key component of a cellular emergency response system to induce cell growth arrest or apoptosis (El-Deiry et al., 1993; Levine, 1997). Absence of p53 or p53 function markedly attenuates radiation- and drug-induced apoptosis in a variety of murine and human cell systems (reviewed in Eastman, 2004; Scherr, 2004). Further, p53-dependent apoptosis and chemosensitivity may require caspase activation in some cell systems (Schuler et al., 2000; Wu and Ding, 2002). Imbalance in favor of cell survival enables tumor progression and resistance to anticancer drugs. Thus, the anti-apoptotic protein, Bcl-2, can suppress p53-dependent apoptosis in HCT116 human colorectal cancer cells, and silencing of Bcl-2 induces massive p53-dependent apoptosis in absence of genotoxic drugs necessary to activate p53 (Jiang and Milner, 2003).

The plant *Pistacia lentiscus* L. var. *chia* grows particularly and almost exclusively in the South region of Chios Island, Greece, and produces a resin, known as Chios mastic gum (CMG). Only a few constituents have been identified from CMG (Papageorgiou et al., 1997); moreover, there is no report on anti-cancer activities of CMG. In this report, we demonstrate that a 50% ethanol CMG extract induces apoptotic death of human colon cancer cells *in vitro* and this process correlates with, but it is not dependent on caspase activation.

Materials and methods

Chemicals/reagents and preparation of gum extracts

Dry Chios mastic resin (gum) was provided by a producer of this product, Chios Island, Greece. Absolute ethanol was purchased from Florida Distillers (Lake Alfred, FL). Cell culture media, RPMI 1640, heat-inactivated bovine serum, trypsin, and antibiotics solutions were purchased from Mediatech, Inc. (Herdon, VA). Hard-dry CMG resin was pulverized to a fine powder with the aid of a kitchen mixer, and identical amounts of the powder were mixed with equal volumes of de-ionized water (Extract I), 25% ethanol in water (Extract II), 50% ethanol in water (Extract III) or 100% ethanol (Extract IV). The mixtures were continually agitated by vertical rotation for 24 h at room temperature, centrifuged to separate soluble (supernatant) from insoluble (pellet) material, and the soluble material was transferred into polypropylene tubes and stored at room temperature in the dark.

Cells and treatments

The HCT116 human colon cancer cell line expressing p53 (HCT116/p53$^{+/+}$) and a clone lacking p53-expression (HCT116/p53$^{-/-}$) were donated by Dr. Bert Vogelstein (Bunz et al., 1999). A clone of HCT116 cells over-expressing Bcl-2 (HCT116/Bcl-2) was developed in our laboratory. A clone of HCT116 cells transfected with FADD dominant-negative plasmid, and thus not expressing functional FADD (clone HCT116/DN.FADD) was also developed in our laboratory using a plasmid donated by Dr. Andreas Strasser (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). All cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine serum and antibiotics. The cell cultures were maintained and propagated in a humidified 37 °C incubator with a 5% CO_2 atmosphere.

Flow cytometry, immunoblotting, and transmission electron microscopy

CMG-induced perturbations in the cell cycle and apoptosis were monitored with the aid of an EPICS XL flow cytometer (BeckmanCoulter, Miami, FL), equipped with the Multicycle AV program for cell cycle analysis (Phoenix Flow Systems, San Diego, CA). The cell samples were treated with RNAse and stained with propidium iodide (PI) immediately prior to analysis. We chose to use the PI methodology over the TUNEL and Annexin-V methodologies for flow cytometry analysis because the TUNEL and Annexin-V methods detect early stages of apoptosis, whereas the PI method detects sub-G_1 cells, that is, cells at late and irreversible apoptotic stages (Pozarowski and Darzynkiewicz, 2004; Span et al., 2002; Tao et al., 2004; Telford et al., 2004; Tuschl and Schwab, 2004; Wilkins et al., 2002). We have used this methodology in previously reported studies (Hu et al., 2003; Pantazis et al., 1993, 2000).

Whole cell extracts were prepared and subjected to immunoblot analysis as described (Chatterjee et al., 2001). The antibodies used in this report recognized human caspase-8 (Sigma–Aldrich, St. Louis, MO);

caspase-3, caspase-9, p53, p21 (Santa Cruz Biotechnology, Santa Cruz, CA); and PARP (BioMol, Plymouth Meeting, PA).

For electron microscopy studies, cells growing at exponential phase and attached to culture dishes received CMG Extract III to a final ethanol concentration of 0.5% or ethanol alone to 0.5% (control culture), and attached and/or detached cells were collected in the culture media, fixed in 3% glutaraldehyde as described (Coelho et al., 1998), post-fixed in 1% osmium, dehydrated in ethanol series with en-block staining with uranyl acetate at the 50% step, and then embedded in Spurr's resin. Thin sections were viewed under a Philips-300 electron microscope at 60 KV.

Results and discussion

CMG induces cell growth inhibition and apoptosis

To investigate whether CMG inhibits growth of HCT116 cells, identical cell cultures received Extracts II, III and IV to a final concentration of 0.5% ethanol, except for Extract I, which was added to a volume equal to that of Extract II. Control cultures received either no additive or ethanol alone to 0.5%. After 48 h, the cells attached to the culture dish were stained and photographed (Fig. 1a). No visible growth inhibition was observed in the cultures treated with Extract I (well B) as compared to controls (A, F); but growth was increasingly inhibited in cultures treated with Extract II (well C); Extract III (well D), and Extract IV (well E). In repeated experiments, however, addition of Extract IV to the culture (to obtain 0.5% ethanol concentration) resulted in extensive visible precipitation of insoluble material (not shown). From these observations we chose to use Extract III for subsequent routine experimentation. To further assess the growth-inhibitory activity of the CMG Extract III, identical cell populations were concurrently exposed to various concentrations of Extract III as described in the legend of Fig. 1b. It is apparent that CMG concentrations contained in wells C, D and E prevented growth of the cells when compared to untreated control (well A) or ethanol-treated (well F) cells (Fig. 1b). It is also apparent that the CMG concentration in well C was highly effective in inhibiting cell growth and/or killing cells at 96 h.

To investigate whether CMG induces perturbations in the cell cycle, identical cell cultures received Extract III to final concentrations of 0.2% and 0.6% for 72 h, and the cultures were then analyzed by flow cytometry (Fig. 2). Control cultures received no additive or 0.6% ethanol alone. Apoptotic (AP) cells were absent in the control cultures (histograms A, D), but present in the CMG-treated cultures (histograms B, C). In conclusion,

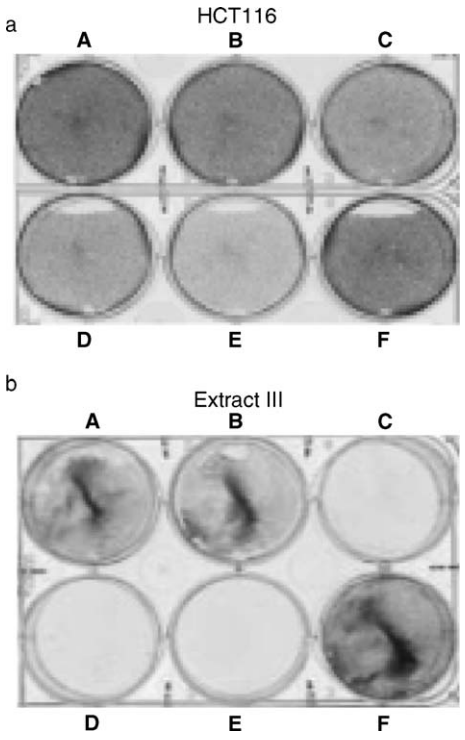
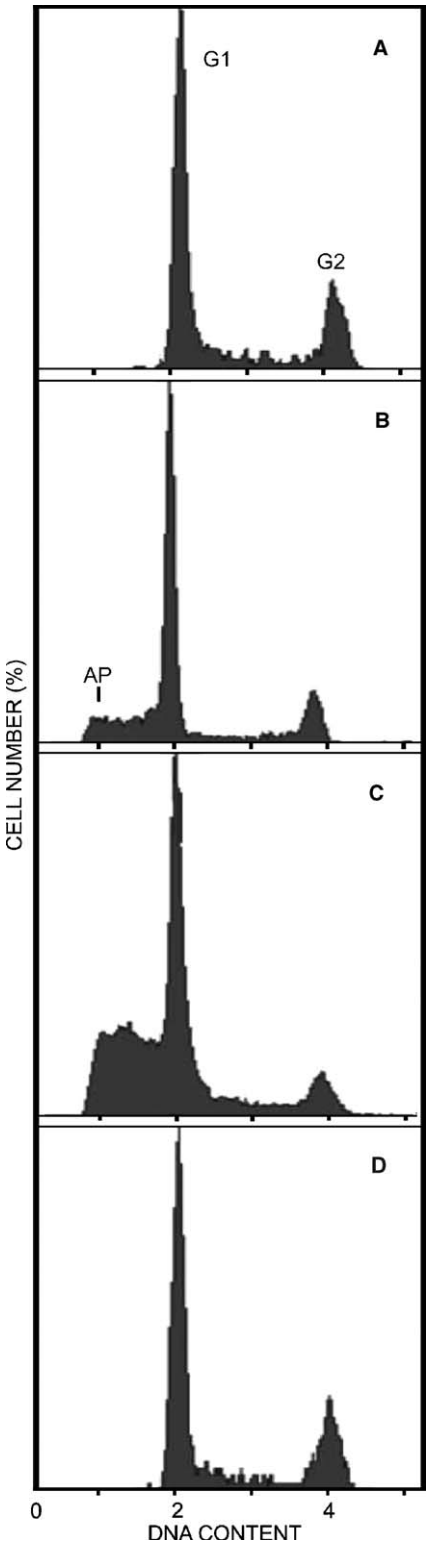


Fig. 1. CMG inhibits growth of HCT116 cells. (1a) Identical cell populations were seeded in a 6-well plate and after 24 h received various ethanol CMG extracts so that the final ethanol concentration in each culture (well) was 0.5%. The plate was incubated for an additional period of 48 h; attached cells were then stained with crystal violet and photographed. Well A, untreated; B, +Extract I; C, +Extract II; D, +Extract III; E, +Extract IV; and F, +ethanol alone (control). (1b) Identical cell populations were seeded in a 6-well plate in media containing various volumes (i.e., CMG concentrations) of Extract III so that the final ethanol concentrations were 0.1% (well B), 0.2% (well C), 0.3% (well D) and 0.5% (well E). Control cultures received no additive (control no. 1, well A) or 0.5% ethanol alone (control no. 2, well F). The plate was incubated for 96 h; attached cells were then stained with crystal violet and photographed.

these results indicate that CMG exerts a concentration-dependent apoptotic effect by directly or indirectly inducing cell-arrest at G_1, followed by DNA damage as indicated by the presence of cells with DNA content less than that of G_1 cells. It should be noted that upon microscopic examination, the cultures contained over 96% attached cells (A, D), 85–90% attached cells (B), and 100% non-attached or detached cells (C).

We further investigated whether cell cycle perturbations are dependent on the duration of CMG treatment. Following pilot experiments, cell cultures received Extract III/ethanol to 0.5% and the cells were collected at various periods of CMG-treatment and analyzed by flow cytometry (Fig. 3). Treatment for 24 and 48 h resulted in cessation of cell proliferation, as assessed by the absence of cells in S-phase (area between G_1 and G_2),

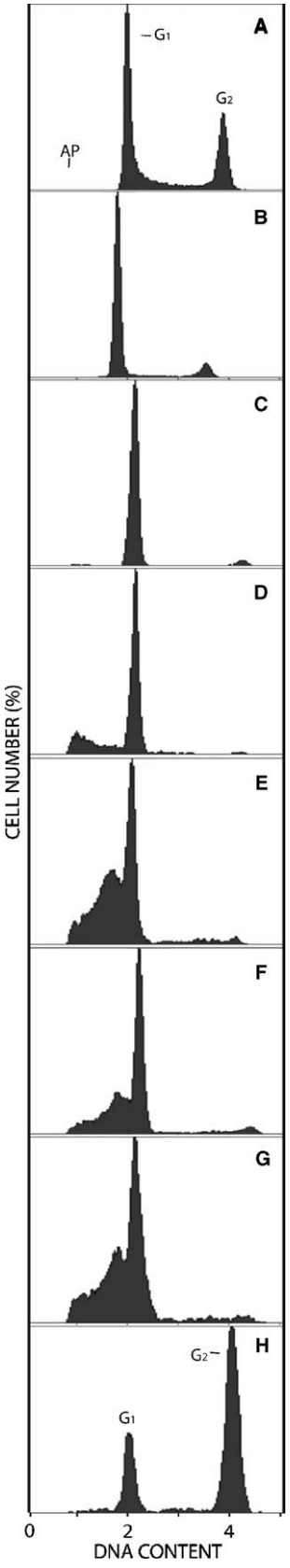
a decrease in the G_2-cell population, and a concomitant increase in the G_1-cell fraction (histograms B and C). AP cell fraction was first detected at 72 h of treatment (histogram D), and continued to increase as CMG treatment continued for 96, 120 and 144 h (histograms E, F, G, respectively). To further confirm CMG-induced cell accumulation at G_1, the cells were treated with camptothecin (CPT), an anticancer drug that results in accumulation of these cells in G_2 (histogram H, and Han et al., 2002). In conclusion, cell accumulation at G_1 occurred during the first 48 h of treatment, while apoptotic cells appeared after their detachment. This form of induction of apoptosis in cells after the loss of adhesion to the substrate has been termed "anoikis", and is triggered by loss of cell-matrix interactions (Jäättelä and Tschopp, 2003; Lockshin and Zakeri, 2004).



CMG-induced cell death is independent of p53, Bcl-2 and FADD

The absence of p53 or p53 function can dramatically attenuate radiation- and drug-induced apoptosis in various cell systems (Eastman, 2004; Scherr, 2004). Therefore, we investigated whether p53 is involved in CMG-induced apoptosis in HCT116 cells. Parental HCT116 (p53-expressing) and HCT116/p53$^{-/-}$ (lacking p53 expression) cells were exposed to the same CMG-concentration, and then subjected to flow cytometry analysis (Fig. 4). Controls included untreated cells growing exponentially (A, F) and untreated cells that were allowed to reach confluence, i.e., G_1-arrest (E, J). The histograms of both cell types obtained at various periods of CMG-treatment indicated that the presence of p53 in HCT116 cells does not increase susceptibility or confer resistance to CMG-treatment. This conclusion was further confirmed by demonstrating that p53 and p21 are expressed in untreated cells (Fig. 5, lane A), but their expression is down-regulated after 24 h treatment with CMG (lane B), that is, at the time the cells accumulate at G_1 as shown by flow cytometry (Fig. 4, histogram B). Down-regulation of p53 and p21 is also observed in untreated, confluent G_1-arrested cells (lane F), indicating that down-regulation of p53 and p21 is associated specifically with cell arrest at G_1. In agreement, practically no p53 and p21 was expressed in cells exposed to CMG for 72, 120 and 144 h (lanes C, D, E, respectively). By contrast, both p53 and p21 were over-expressed in CPT-treated, G_2-arrested cells (lane G) in agreement with a previous report (Han et al., 2002).

Fig. 2. Detection of apoptosis by flow cytometry. HCT116 cells received Extract III volumes that resulted in ethanol concentrations of 0.2% (histogram B), and 0.6% (histogram C) in the cultures. Control cultures received no additive (histogram A) or 0.6% ethanol alone (histogram D). The cultures were then incubated for 72 h, harvested and subjected to flow cytometry analysis. The histograms were derived from flow cytometry analysis of a pool of attached and non-attached cells. G_1, G_1+G_0 cells; G_2, G_2+M; and AP, apoptotic cells.



Therefore, we conclude that CMG-induced G_1 arrest, but not apoptosis, is associated with down-regulation or lack of expression of p53 and p21 in HCT116 cells.

Subsequently, we investigated whether CMG-induced apoptosis is mediated by the mitochondrion-dependent pathway, which is regulated by the quantitative ratio of pro- to anti-apoptotic proteins of the Bcl-2 family. In this regard, silencing of the anti-apoptotic protein, Bcl-2, induces massive p53-dependent apoptosis in HCT116 cells in the absence of genotoxic drugs necessary to activate p53 (Jiang and Milner, 2003). Conversely, over-expression of Bcl-2 in HCT116 cells would increase the resistance of these cells to CMG-induced apoptosis. To investigate this possibility, we compared the sensitivity of HCT116 and HCT116/Bcl-2 (Bcl-2-over-expressing) cells to CMG-treatment. The histograms (Fig. 4) derived from CMG-treated HCT116/Bcl-2 cells (K through O) are not significantly different from CMG-treated HCT116 cells (A through E), indicating that over-expression of Bcl-2 in confers no significant increase in resistance to CMG-induced apoptosis.

We finally investigated whether CMG induces apoptosis via the death receptor-dependent pathway. Execution of this pathway requires that pro-caspase-8 interacts with the adaptor protein, Fas-associated death domain (FADD), recruited and bound to activated death receptors to form a death-inducing signaling complex (DISC). Therefore, absence of FADD will result in absence of caspase-8 activation and lack of execution of the death-receptor pathway. For this investigation, we analyzed CMG-induced apoptosis in HCT116 and HCT116/DN.FADD cells using flow cytometry. The histograms of untreated and CMG-treated HCT116 (A through E) and HCT116/DN.FADD (P through T) cells indicate similar responses of these two cell types to CMG at specific time-points of the treatment (Fig. 4, compare B with Q, C with R, and D with S). Therefore, we have concluded that CMG-induced apoptosis is not mediated by the death-receptor pathway.

CMG-induced cell death is associated with caspase-3 activation and PARP degradation

It has been reported that cell death may occur via apoptotic-like pathways independent of caspases

Fig. 3. Induction of apoptosis is time-dependent in CMG-treated cells. Identical cell cultures received CMG Extract III to a final concentration of 0.5%, and then incubated for 24 h (B), 48 h (C), 72 h (D), 96 h (E), 120 h (F), and 144 h (G) before the cells were harvested and subjected to flow cytometry analysis to detect cell cycle perturbations and apoptosis. Controls included exponentially growing untreated cells (A), and cells treated with 25 nM CPT for 48 h (H).

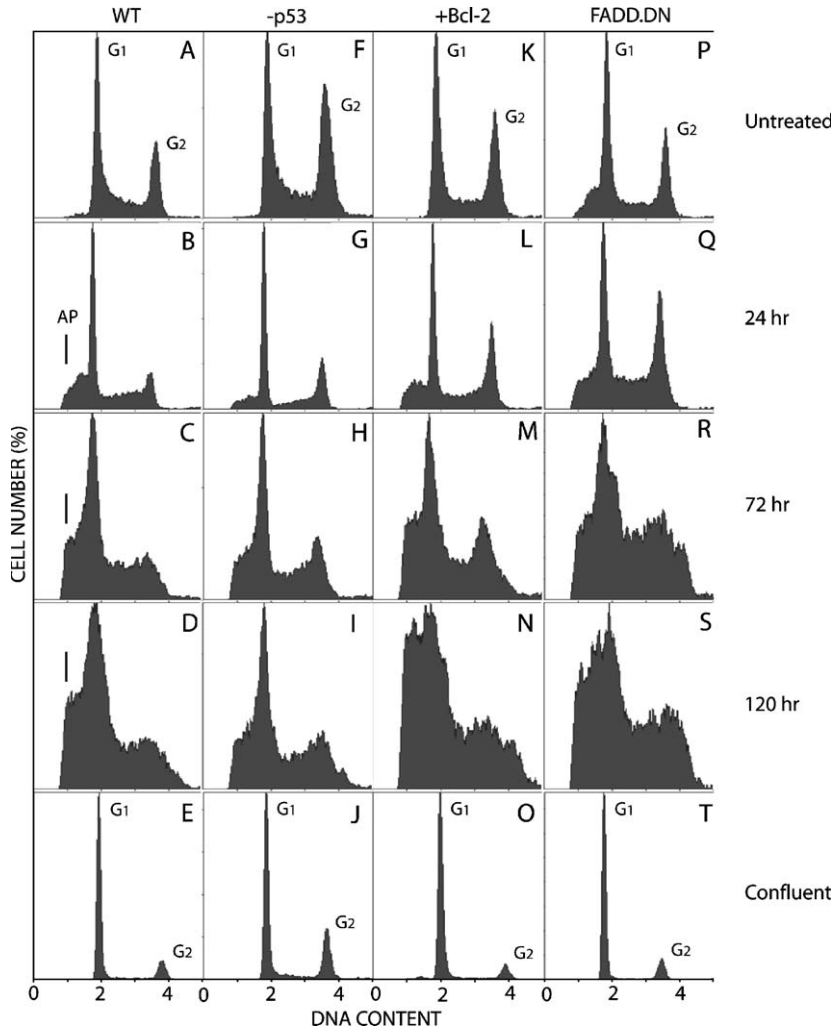


Fig. 4. Flow cytometry analysis of HCT116, HCT116/p53$^{-/-}$, HCT116/Bcl-2 and HCT116/DN.FADD cells treated with CMG Extract III. Identical cultures of each cell type were exposed to CMG Extract III for various periods on time before the cells were harvested and subjected to flow cytometry analyses. The histograms shown were derived from untreated exponentially growing cells (A, F, K, P), cells treated with CMG for 24 h (B, G, L, Q), 72 h (C, H, M, R), 120 h (D, I, N, S), and untreated cells that were arrested at G_1 by reaching confluence (E, J, O, T). HCT116, A through E; HCT116/p53$^{-/-}$, F through J; HCT116/Bcl-2, K through O; and, HCT116/DN.FADD, P through T.

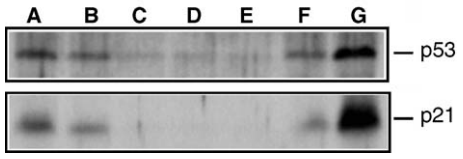


Fig. 5. Down-regulation of p53 and p21 expression in CMG-treated cells. Depending on the treatment, attached, attached + non-attached, or non-attached cells, were processed to obtain whole protein extracts and then 50 µg of protein/sample were subjected to Western immunoblot analysis of p53 and p21. Extracts were prepared from untreated exponentially growing HCT116 cells (A), cells treated with CMG for 24 h (B), 72 h (C), 120 h (D) and 144 h (E), and confluent G_1-arrested (F) and CPT-treated G_2-arrested (G) cells.

(Jäättelä, 2004; Lockshin and Zakeri, 2004). In this regard, we investigated whether induction of apoptosis, detected by flow cytometry, is dependent on caspase activation in CMG-treated HCT116 cells. For this study, we detected the presence of proteolytic degradation (i.e., activation) of pro-caspase-3 in cells treated with CMG for 72 h. Increased processing of pro-caspase-3 was observed after treatment with various CMG contents: that is, Extract III volumes that resulted in 0.1% (B), 0.2% (C) and 0.5% (D), but not in control untreated (A) or ethanol-treated (E) cells (Fig. 6). The results shown in Fig. 6 were derived upon analysis of identical amounts of total cell protein as indicated by the presence of similar amounts of the control protein, β-actin, in all samples.

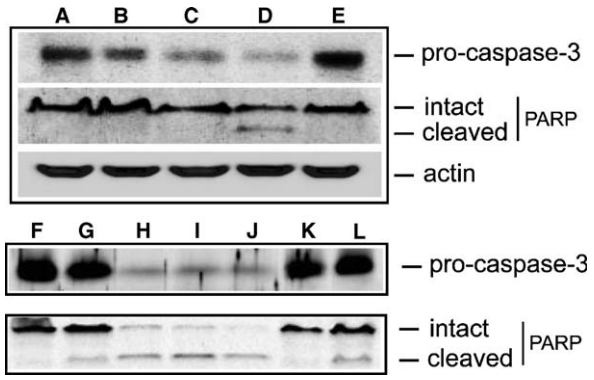


Fig. 6. CMG-induced apoptosis is associated with caspase activation. Whole cell protein extracts were subjected to Western blot analysis using specific antibodies to pro-caspase-3, PARP, and β-actin. Exponentially growing HCT116 cells received Extract III to final ethanol concentrations of 0.1% (B), 0.2% (C), and 0.5% (D). Control cells received no additive (A) or were exposed to 0.5% ethanol alone (E). Control and CMG-treated cells were incubated for 72 h. In another experiment, exponentially growing cells were left untreated (F) or were treated with Extract III/0.5% ethanol for 24 h (G), 72 h (H), 120 h (I), and 144 h (J). Untreated cells were also allowed to reach confluence and become G_1-arrested (K) or were treated with 25 nM CPT for 144 h to become G_2-arrested and senescent (L).

Proteolytic processing of inactive pro-caspase-3 to activated caspase-3 is a late event of the apoptotic pathway, and is followed by caspase-3-targeted and cleavage of vital macromolecules including the enzyme poly(ADP-ribose)polymerase, PARP (Krammer and Debatin 2004; Strasser et al., 2001). We investigated the cleavage of PARP in untreated and CMG-treated HCT116 cells by detecting the 116 kD-intact and 85 kD-cleaved PARP identified by Western blotting using an antibody that recognizes these two macromolecules (Fig. 6). Cleaved PARP was readily detected in cells exposed to CMG/0.5% ethanol (lane D), but not in untreated (lane A) and ethanol treated (lane E) control cells. Also, no cleaved PARP was present in cells treated with CMG/0.1% ethanol (lane B) and CMG/0.2% ethanol (lane C) for 72 h, apparently because the CMG concentrations were inadequate to generate visible PARP cleavage in 72 h. We further investigated the onset of pro-caspase-3 degradation (i.e., activation) and PARP degradation in HCT116 cells treated with CMG/0.5% ethanol for various periods of time (Fig. 6, lanes F–L). Nearly identical amounts of pro-caspase-3 and PARP were present in exponentially grown untreated and 24 h-treated cells (lanes F, G), that is, cells attached to the substrate. However, a dramatic pro-caspase-3 and PARP reduction, as a result of specific degradation, was detected at 72, 120 and 144 h of treatment in cells that were detached from the substrate (lanes H, I, J). No pro-caspase-3 degradation was apparent in untreated confluent and CPT-arrested senescent cells (lanes K, L, respectively) that were also attached to the substrate. Further, cleaved 85-kD PARP was absent in untreated confluent G_1-arrested cells (lane K), whereas a small amount of the 85-kD PARP product was present in CPT-treated G_2-arrested cells, apparently because of spontaneous death of senescent HCT116 cells after 144 h of CPT-treatment (lane L). In conclusion, processing of pro-caspase-3 and PARP increased as a function of the treatment period and resulted in complete conversion of intact 115-kD PARP to 85-kD cleaved product at 144 h of treatment (lane J).

Activation of caspases-8 and -9 upon CMG-induced cell death

Flow cytometry results indicated that Bcl-2 overexpression or an impairment in the death receptor-dependent apoptotic pathway does not protect HCT116 cells from CMG-induced apoptosis (Fig. 4). On the other hand, CMG induced activation of caspase-3 (Fig. 6), a late enzymatic event in the mitochondrion- and/or death receptor-dependent pathways. Further, caspase-9 was required for p53-independent apoptosis in a human ovarian cancer cell line (Wu and Ding, 2002). Therefore, we investigated the processing (i.e., activation) of caspases-8 and -9 that have been associated with the pathways dependent on death receptors and mitochondria, respectively. Processing of pro-caspases-8 and -9 was compared in CMG-treated HCT116, HCT116/DN.FADD and HCT116/Bcl-2 cells (Fig. 7). Only the intact pro-caspase-8 doublet was present in untreated HCT116 cells (lane A), whereas a small amount of p18, a product of pro-caspase-8, was detected at 24 h of treatment (lane B), but the p18 amount increased at 72 h of treatment (lane C). Increases in p18 were concomitant with decreases in pro-caspase-8. A similar processing pattern of pro-caspase-8 was also detected in HCT116/Bcl-2 cells treated with CMG for 24 and 72 h (lanes D,

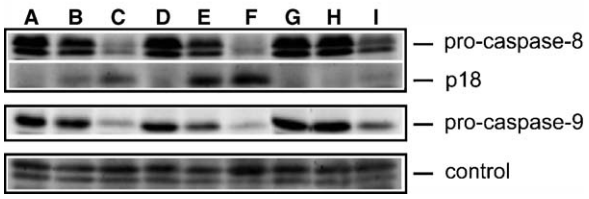


Fig. 7. Detection of caspase-8 and caspase-9 by Western immunoblotting. Whole cell protein prepared from untreated and CMG/Extract III-treated cells were subjected to analysis for detection of pro-caspase-8 and -9 processing. Lanes A, B, C, HCT116 cells; lanes D, E, F, HCT116/Bcl-2 cells; lanes G, H, I, HCT116/FADD.DN cells. Untreated cells, A, D, G; cells treated with CMG for 24 h (B, E, H), and 72 h (C, F, I). Non-specific intracellular proteins served as control.

E, F). Further, a much less extensive processing of pro-caspase-8 was detected in CMG-treated HCT116/DN.FADD cells (lanes G, H, I). In a parallel experiment using the same cell protein extracts, similar patterns of pro-caspase-9 processing, i.e., a decrease, was observed in CMG-treated HCT116 and HCT116/Bcl-2 cells, but a less extensive processing in CMG-treated HCT116/DN.FADD cells (Fig. 7). However, inadequate processing (i.e., insufficient activation) of both pro-caspase-8 and pro-caspase-9 in HCT116/DN.FADD cells does not appear to confer significant resistance to CMG-induced death as assessed by flow cytometry (Fig. 4). It should be noted that the specific processing of pro-caspases-8 and -9 was confirmed by the absence of quantitative changes in the amounts of other cellular proteins assured that equal protein amounts were subjected to analysis (Fig. 7). In conclusion, it appears that induction of apoptosis in CMG-treated cells correlates with activation of, but is not dependent on, caspases-8 and -9.

Subcellular morphological features of CMG-treated cells

In addition to flow cytometry and biochemical analyses, CMG-treated cells were examined by electron microscopy for ultrastructural changes in major organelles after treatment with CMG/0.5% ethanol for 72 h (Fig. 8). Control cells were treated with 0.5% ethanol alone. Compared to untreated control (A, B), CMG-treated cells exhibit advanced stages of degeneration including loss of the smooth continuity of the cell membrane (cm), appearance of lobulated nuclei (nu), heterochromatin accumulation along the inner surface of the nuclear envelop (ne), increased and pronounced nucleolar material (nm), disintegration or loss of organization of mitochondria (mi) structures and network and the appearance of numerous vacuoles (v) of various sizes as well as secretory vesicles (sv). However, the cells are not fragmented in a classical apoptotic manner. Some of the organelle changes have been associated with the well-studied forms of apoptosis, whereas other changes appear to be associated with other forms of cell death (Brivida et al., 2002). Similar observations of non-typical apoptotic morphological features have been described for other cell systems (reviewed in Jäättelä and Tschopp, 2003; Lockshin and Zakeri, 2001). It is also possible that CMG-induced death of HCT116 cells involves mechanisms overlapping with or unrelated to classical apoptotic pathways (Abraham and Shaham, 2004; Assuncao Guimaroes et al., 2004; Jäättelä and Tschopp, 2003). CMG treatment therefore induces death of HCT116 cells with concomitant or preceding appearance of morphological features that suggest activation of classical apoptotic and non-apoptotic mechanism(s).

Long-term effect of CMG-treatment

Finally, we investigated the fate of CMG-detached cells after removal of CMG from the culture. In this study, HCT116 cells were treated with CMG-Extract III (0.5% ethanol concentration in the culture) for 72 h to

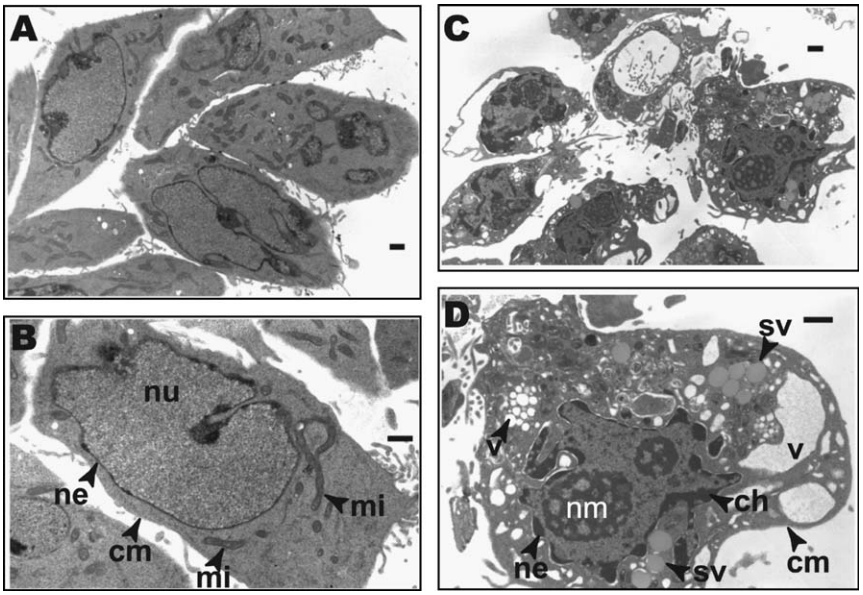


Fig. 8. Electron microscopy of HCT116 cells before and after treatment with CMG. Untreated (A, B) and CMG-treated (C, D) cells were photographed at low (A, C) and high (B, D) magnification. Treatment was for 72 h. Cell organelles shown are: cell membrane (cm); nucleus (nu); chromatin (ch); nuclear envelope (ne); nucleolar material (nm); mitochondrion (mi); vacuole (v); and, secretory vesicle (sv). Scale bar, 1 μm.

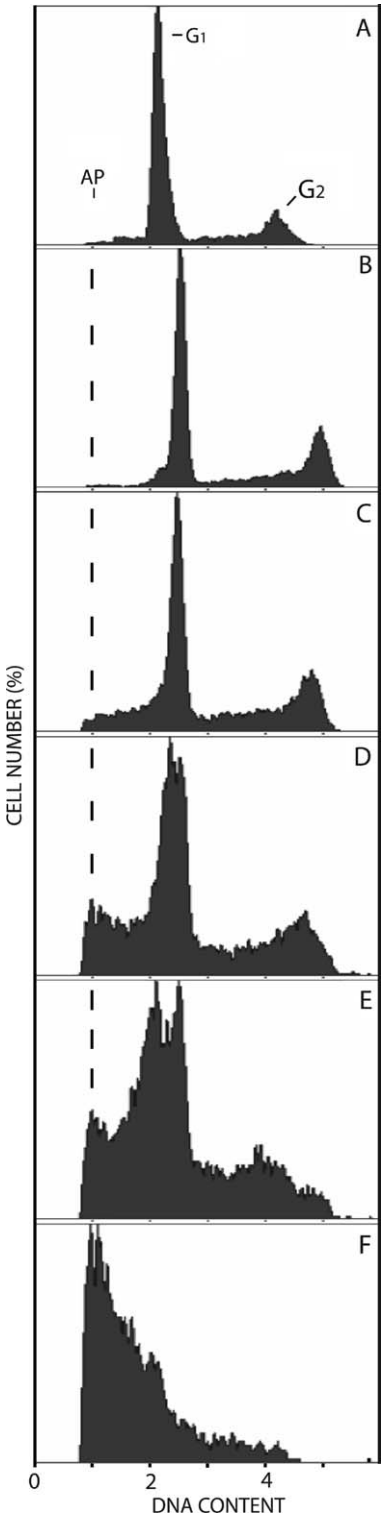


Fig. 9. Cell cycle analysis of CMG-released cells. Following 72 h of CMG-treatment, detached HCT116 cells were transferred into CMG-free media and incubated at 37 °C for various periods of time before they were harvested and subjected to flow cytometry analysis for detection of cell cycle perturbations and apoptosis. CMG-treated cells were placed in CMG-free medium for 0 (A), 2 (B), 4 (C), 6 (D), 8 (E) and 12 (F) days.

allow the cells to detach from the substrate. Subsequently, the detached cells were harvested by gentle centrifugation, washed and suspended in fresh CMG-free media, and divided into six equal volumes (A through F), presumably containing identical cell populations. CMG-released cells in volume A were processed and stored for flow cytometry analysis, whereas volumes B through F were placed in culture dishes and incubated at 37 °C. The cultures received three ml of fresh media every two days, and the cells were collected at various periods of CMG-release for flow cytometry analysis (Fig. 9). As observed earlier (Figs. 3 and 4), CMG treatment for 48–72 h resulted in extensive accumulation of cells at G_1 (Fig. 9, histogram A). Two-day released cells were still arrested at G_1, while a negligible AP-fraction was present (B). The AP-fraction increased in the culture as the release period continued for 4 (C), 6 (D) and 8 (E) days. Finally, the 12-day released culture contained over 90% apoptotic cells (F). These results, taken collectively, indicate that after three days of CMG treatment, the presence of CMG is not required for the detached HCT116 cells to continue dying; that is, the cells have committed themselves to apoptosis. CMG treatment therefore causes a cascade of cellular events ultimately resulting in the interruption of matrix adhesion and triggering (anoikis form) irreversible cell death. This could have practical applications if CMG exhibits the same anticancer activities *in vivo*.

At present, we are in the process of identifying the constituent(s) in the 50% ethanolic CMG-Extract III that result in G_1-arrest, loss of matrix adhesion and caspase-dependent or independent death in HCT116 cells. To identify the active component(s) in Extract III, dry CMG has been and will be subjected to sequential fractionation and testing of fractions *in vitro*. Active fractions will be analyzed chemically to identify their components. Our findings reported here therefore serve only as a prelude to *in vitro* and *in vivo* studies that may lead to the verification of the efficacy of the anticancer activities of CMG against various human cancers and the identification of the active CMG components.

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