

Caspase-8 and Apoptosis-inducing Factor Mediate a Cytochrome *c*-independent Pathway of Apoptosis in Human Colon Cancer Cells Induced by the Dietary Phytochemical Chlorophyllin¹

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ABSTRACT

Chlorophyllin (CHL), an antimutagenic and anticarcinogenic water-soluble derivative of chlorophyll, was recently found to be highly effective as a chemopreventive agent in a high-risk population exposed unavoidably to aflatoxin B₁ in the diet (P. A. Egner *et al.*, Proc. Natl. Acad. Sci. USA, 98: 14601–14606, 2001). The current study examined the response of HCT116 human colon cancer cells to CHL treatment. Cells exposed to concentrations in the range 0.0625–0.5 mM CHL underwent growth arrest and apoptosis after 24 h, with the formation of a sub-G₁ peak in the attached cell population and nuclear condensation in the floating cell population. There was a concentration-dependent attenuation of mitochondrial membrane potential ($\Delta\psi_m$) without the release of cytochrome *c* or activation of the caspase-9/caspase-3/poly(ADP-ribose) polymerase pathway. However, apoptosis-inducing factor was released from mitochondria into the cytosol and translocated to the nucleus, leading to concentration-dependent cleavage of nuclear lamins. The upstream mediators of this CHL-induced apoptosis pathway were identified as caspase-8/caspase-6 and truncated Bid, acting in conjunction with other proapoptotic members of the Bcl-2 family, such as Bak. These findings suggest that CHL might trigger apoptosis via interaction with putative “death receptors” in the plasma membrane of cancer cells, leading to initial cleavage of procaspase-8 and activation of subsequent downstream events, resulting in the destruction of nuclear lamins. Importantly, E-cadherin and alkaline phosphatase, which are indicators of cell differentiation, were strongly induced at all concentrations of CHL. Thus, in addition to being an effective blocking agent during the initiation phase, these findings support a role for CHL as a suppressing agent and as a possible novel therapeutic strategy directed toward aberrant cell proliferation in the colon.

INTRODUCTION

Apoptosis is a form of programmed cell death characterized by stereotypic morphological changes executed by cysteine-aspartate proteases (caspases) and regulated by the Bcl-2 family of proteins (1). Diverse death signals activate downstream “effector proteases” via one of two broad mechanisms, namely, an extrinsic pathway involving cell death receptors and an intrinsic pathway resulting from alterations at the level of the mitochondrion and involving cytochrome *c* release and activation of the apoptosome (2). In the latter case, cytochrome *c* and Apaf-1, in the presence of ATP or dATP, facilitate the cleavage of procaspase-9 and generate active caspase-9 cleavage products (3). Caspase-9, in turn, activates executioner caspases, such as caspase-3 and caspase-7, via the so-called “caspase cascade” (4). The hierarchical activation of these caspases ultimately results in the cleavage of key nuclear, cytoplasmic, and membrane-associated proteins, as well as the activation of DNases in many cases, leading to the

morphological and biochemical changes characteristic of apoptotic cell death (5, 6). Although these are regarded as central features of apoptosis, several studies have revealed “variations on a theme,” including Bcl-2-regulated caspase activation independent of the cytochrome *c*/Apaf-1/caspase-9 apoptosome (7). In the course of investigating the mechanisms by which dietary phytochemicals inhibit colon carcinogenesis in rodent models and in human colon cancer cells, we identified a pathway of cell death involving the release of AIF³ from mitochondria without the concomitant release of cytochrome *c*.

The phytochemical that induced this cytochrome *c*-independent pathway of apoptosis, CHL, is a clinically used water-soluble derivative of chlorophyll. Chlorophyll and CHL are known to exhibit potent antimutagenic activity *in vitro*, and they have been reported as anticarcinogens in various animal models (8–10). As a “blocking agent,” CHL forms molecular complexes with aromatic carcinogens, such as heterocyclic amines, polycyclic aromatic carbons, and aflatoxins, and reduces carcinogen uptake and bioavailability from the gut (10). Postinitiation mechanisms have received much less attention, although recent work showed that CHL altered the spectrum of β -catenin mutations in carcinogen-induced colon tumors in the rat (11) and that this involved changes in cell proliferation and apoptosis rates in the colonic crypt (12). The ability of CHL to alter tissue homeostasis in the colon suggested an investigation of apoptosis mechanisms in human colon cancer cells.

Previously, chemopreventive agents such as butyrate, salicylate, and vitamin D were shown to induce apoptosis in colorectal cancer cells with the involvement of the cytochrome *c*/Apaf-1/caspases-9 apoptosome (13–16). In marked contrast, the present investigation shows that CHL induces apoptosis in HCT116 cells without the release of cytochrome *c* from mitochondria or activation of the Apaf-1/caspase-9 pathway. As an alternative mechanism, CHL activated a pathway involving caspase-6 and the release of AIF from mitochondria, resulting in the cleavage of nuclear lamins. CHL also induced changes in the expression of Bcl-2 family proteins, including tBid and Bak, and apoptosis occurred subsequent to the induction of colonic cell differentiation, as evidenced by the increased expression of differentiation markers such as E-cadherin and ALP.

MATERIALS AND METHODS

Cell Culture. The human colorectal cancer cell line HCT116 was obtained from the Cell Culture Facility Core of the Environmental Health Sciences Center, Oregon State University. Cells were grown in McCoy’s 5A (Life Technologies, Inc.) containing 10% fetal bovine serum (batch-selected; Life Technologies, Inc.), glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μ g/ml; Sigma, St. Louis, MO). Cells were grown in T25 flasks and routinely passaged using 0.1% trypsin (Difco)/0.1% EDTA (Fisher).

Treatment of Cells with CHL. The CHL used in these studies was from Sigma. Concentrations were adjusted for total chlorin content (53%), of which the major components were chlorin-e₆ (63%) and isochlorin-e₄ (16%). Chlor-

Received 1/31/02; accepted 1/15/03.

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¹ Supported in part by NIH grants CA65525 and CA80176, and NIEHS Center grant P30 ES00210.

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³ The abbreviations used are: AIF, apoptosis-inducing factor; CHL, chlorophyllin; Rho-123, rhodamine 123; PARP, poly(ADP-ribose) polymerase; tBid, truncated Bid; ALP, alkaline phosphatase; FACS, fluorescence-activated cell-sorting; PMSF, phenylmethylsulfonyl fluoride.

ins were identified using high-performance liquid chromatography methods reported elsewhere (17, 18). Stock solutions of 40 mM CHL in double-distilled water were made immediately before use, and serial dilutions were performed in the cell culture media just before addition to the cells. All of the solutions containing CHL were protected from light. Cells were seeded at a density of 1×10^6 cells/T25 flask, and they were treated with CHL 3 days later, when each of the flasks contained a similar number of cells, and cells were in the exponential growth phase. Cultures were treated in triplicate with CHL, and the cells were harvested after 24 h, unless stated otherwise.

Identification of Apoptosis. The induction of apoptosis by CHL was determined by morphological and biochemical criteria, as follows. Acridine orange/ethidium bromide dual staining was used to identify apoptotic cells by staining the condensed chromatin. Unfixed cells were stained with 5 $\mu\text{g}/\text{ml}$ acridine orange together with 5 $\mu\text{g}/\text{ml}$ ethidium bromide in PBS for 10 min and then viewed by fluorescence microscopy. Acridine orange stains early-stage apoptotic cells, whereas late-stage apoptotic cells that have lost membrane permeability stain with ethidium bromide. Ethidium bromide also allows for the identification of cells that are necrotic, due to loss of membrane permeability without the presence of condensed chromatin. At least 200 cells were scored for each sample in three independent experiments. Cells were scored as positive based on visual evidence of nuclear condensation, membrane blebbing, or cytoplasmic condensation. The floating cells were also assessed using a TACS Apoptotic DNA Laddering kit, according to the manufacturer's instructions (Trevigen, Gaithersburg, MD). Briefly, genomic DNA was isolated from 10^6 floating cells and loaded onto a 1.0% TreviGel 500 gel in a $1 \times$ TAE buffer [40 mM Tris, 5 mM sodium acetate, and 1 mM EDTA (pH 8.1)], containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. The gel was run at 100 V until the bromophenol blue dye front reached the lower third of the gel. As a positive control for DNA laddering, HCT116 cells were treated with 4 mM sodium butyrate (Sigma) for 24 h.

Cell Cycle Analysis. One million adherent cells were trypsinized and washed in PBS, fixed in 70% ethanol, and stored at -20°C overnight. In preparation for cell cycle analysis, the cells were pelleted, resuspended in 1 ml of PBS containing 0.2 mg/ml propidium iodide (Sigma) and 0.15 $\mu\text{g}/\text{ml}$ RNase (Boehringer Mannheim), and incubated at 37°C for 30 min in the dark. Cells were then left overnight at 4°C in the dark, before analysis on a fluorescence-activated cell sorter (Coulter FACS). Cells were excited with a single 488 nm argon laser, and the fluorescence was detected through a 585 ± 22 nm filter. Linear red-orange fluorescence (FL3) data were collected in list format to 10,000 total events, and the distribution of cells within the cell cycle was determined using a MultiCycle software program.

Subcellular Fractionation and Immunoblot Analysis. Nuclear protein extraction was performed as follows. Briefly, PBS-washed cells were resuspended in extraction buffer [10 mM Tris-HCl (pH 7.6), 10 mM KCl, and 5 mM MgCl_2] with protease inhibitors (0.2 mM PMSF, 10 $\mu\text{g}/\text{ml}$ pepstatin A, and 10 $\mu\text{g}/\text{ml}$ leupeptin). After incubation on ice for 10 min, cells were lysed by digitonin (Sigma-Aldrich). Nuclei were removed by centrifugation, and nuclear protein was extracted with radioimmunoprecipitation assay buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, and 1 mM DTT] supplemented with protease inhibitors [30 $\mu\text{l}/\text{ml}$ aprotinin, 10 $\mu\text{l}/\text{ml}$ leupeptin, and 10 $\mu\text{l}/\text{ml}$ PMSF (Sigma)]. For cytochrome *c* determination, PBS-washed cells were lysed in isotonic buffer A (10 mM HEPES, 0.3 M mannitol, and 0.1% BSA) plus 0.1 mM digitonin with protease inhibitors. After incubation on ice for 5 min, the cytosolic fraction was collected by centrifugation, and the insoluble fraction was disrupted by sonication in sonication buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 0.5% Tween 2] supplemented with protease inhibitors. For whole cell extractions, samples were washed with 1 ml of PBS, and ice-cold radioimmunoprecipitation assay lysis buffer (2 ml) was added to the cell monolayer followed by gentle scraping on ice. Cell lysates were transferred to a microcentrifuge tube, and a mixture of inhibitors was added [30 $\mu\text{l}/\text{ml}$ aprotinin, 10 $\mu\text{l}/\text{ml}$ leupeptin, and 10 $\mu\text{l}/\text{ml}$ PMSF (Sigma)]. Samples were left on ice for 30 min and centrifuged at $15,000 \times g$ for 15 min. Supernatants were taken for protein quantification, determined as described by Peterson (19). Protein (100 μg) was mixed with 60 μl of gel sample buffer [0.125 M Tris-HCl (pH 6.8; Sigma), 20% (v/v) glycerol, 2% (w/v) SDS, 10% (v/v) β -mercaptoethanol, and 0.25% (w/v) bromophenol blue], and the tubes were placed in a hot plate at 95°C for 5 min.

For SDS-PAGE, the stacking gel was 4.5%, and resolving gels were made

up at 10%, 12.5%, or 15% as required. Gels were run in a buffer solution containing 192 mM glycine (Sigma), 25 mM Tris (Sigma), and 1% SDS and then electroblotted onto Immobilon polyvinylidene difluoride membrane in a transfer buffer of 192 mM glycine (Sigma), 10 mM Tris (Sigma), and 20% (v/v) methanol (Fisher).

Immunoblotting was performed with the following antibodies: (a) Bcl-2 mouse monoclonal clone 65111A (PharMingen), 1:400 dilution; (b) Bak rabbit polyclonal clone G-23 (Santa Cruz Biotechnology), 1:300 dilution; (c) Bax mouse monoclonal clone 66241A (PharMingen), 1:400 dilution; (d) caspase-3 rabbit polyclonal clone 65906E (PharMingen), 1:400 dilution; (e) PARP mouse monoclonal clone 556494 (PharMingen), 1:400 dilution; (f) Bcl- $X_{L/S}$ rabbit polyclonal clone (L-19; Santa Cruz Biotechnology), 1:400 dilution; (g) cytochrome *c* mouse monoclonal clone 556433 (PharMingen), 1:400 dilution; (h) AIF goat polyclonal clone D-20 (sc-9416; Santa Cruz Biotechnology), 1:400 dilution; (i) Bid rabbit polyclonal (#2002; Cell Signalling), 1:500 dilution; (j) lamin A rabbit polyclonal (#2032; Cell Signalling), 1:500 dilution; (k) caspase 6 rabbit polyclonal (#9761; Cell Signalling), 1:500 dilution; (l) caspase 8 mouse monoclonal (#9746; Cell Signalling), 1:500 dilution; (m) caspase 9 rabbit polyclonal (#9505; Cell Signalling), 1:500 dilution; (n) lamin A rabbit polyclonal (#2031; Cell Signalling), 1:500 dilution; and (o) E-cadherin mouse monoclonal clone HECD-1 (Zeneca), 1:1000 dilution. Membranes were incubated in milk block buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 4% nonfat dried milk] for 1 h at room temperature. Incubations with primary antibody were conducted for 4 h at room temperature or overnight at 4°C . Membranes were rinsed in distilled water, washed twice for 10 min in milk block buffer, and then washed twice for 10 min in Tween buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 0.2% Tween 20 (w/v; Sigma)], followed by milk block buffer for an additional 10 min. Secondary antibodies were antimouse or antirabbit horseradish peroxidase-conjugated antibodies (Bio-Rad). Membranes were incubated in the appropriate secondary antibody (1:3000 dilution) for 45 min at room temperature or at 4°C . Membranes were washed for 20 min in milk block buffer and for 10 min in Tween buffer. After rinsing in distilled water, peroxidase activity was detected using ECL Chemiluminescence Reagent Plus (New England Nuclear) according to the protocol supplied with the kit. Equal protein loading was confirmed by Ponceau S staining.

Mitochondrial Transmembrane Potential ($\Delta\psi_m$). Changes in mitochondrial membrane potential were assayed as described elsewhere (20). Briefly, cells were washed twice in PBS containing 1 mM EDTA (PBS-EDTA) and subsequently detached and pelleted in PBS-EDTA buffer. After recovery in complete medium, cells were incubated with 1 μM Rho-123 (Molecular Probes, Eugene, OR) for 30 min at 37°C . Rho-123 is taken up selectively by mitochondria (21, 22), and the extent of relative uptake depends on $\Delta\psi_m$. Cells were washed in ice-cold PBS, filtered, and resuspended in 1 ml of PBS for immediate analysis. Rho-123 fluorescence was excited at 488 nm, and emission at 530 nm was measured using a fluorescence-activated cell sorter (Coulter FACS).

RNA Isolation. Total RNA was extracted using Qiagen Rneasy anion-exchange spin columns and the Total RNA Isolation Kit (Qiagen), according to the instructions of the manufacturer. RNA concentration was quantified spectrophotometrically, and the relative content was further confirmed on ethidium bromide-stained gels. Integrity of the RNA was established routinely by agarose gel electrophoresis (data not shown).

Reverse Transcription-PCR. Synthesis of first-strand cDNA was performed using the SuperScript preamplification system (Life Technologies, Inc.), and 4 μg of total RNA per reaction were reversed transcribed using oligo(dT) as the primer, of which 2 μl from the total volume of 20 μl of cDNA were used for each PCR reaction. Primers were as follows: E-cadherin, 5'-GTTATTCCTCCATCAGCTGCCA-3' (forward) and 5'-CTGGCT-GAGGATGGTGAAGCG-3' (reverse); ALP, 5'-CACCAACGTGGCTAA-GAATGTCATCATG-3' (forward) and 5'-TGGTAGGCCATGCTCTTA-CAGCC-3' (reverse); and GAPDH, 5'-AAGATCATCAGCAATGCTCC-3' (forward) and 5'-CCTGCTTACCACCTTCTGA-3' (reverse).

Reactions (20 μl) were conducted in a thermal cycler (GeneAmp PCR system 9700; Perkin-Elmer, Foster City, CA), initiated by hot start at 94°C for 3 min. Cycling conditions were as follows: 94°C for 30 s; 62°C for 30 s; and 72°C for 30 s; with 27 cycles for E-cadherin and 40 cycles for ALP. All reactions ended with a 5-min elongation step at 72°C . Seven μl of the reaction volume were electrophoresed through a 1.5% agarose gel and stained with

ethidium bromide, and products were visualized using an Alphascreen 1220 Documentation and Analysis System (AlphaInnotech Corp.).

RESULTS

CHL Treatment Reduces the Attached Cell Yield and Induces Apoptosis. CHL was growth inhibitory and induced apoptosis in HCT116 cells (Fig. 1). In previous studies, the majority of colon cancer cells that were shed into the medium, either spontaneously or by treatment with agents such as butyrate and vitamin D (13, 15, 23, 24), had undergone apoptosis. In the present study, there was a concentration-dependent increase in the proportion of floating cells with each CHL addition (Fig. 1A), and this was matched by a concomitant decline in the attached cell yield (Fig. 1B). All concentra-

tions of CHL reported here produced a statistically significant increase in the percentage of floating cells and reduction in attached cell yield ($P < 0.05$). The highest concentration of CHL used in these studies caused 30% of the cells to be floating 24 h after treatment, and the corresponding attached cell yield was 30% of controls. Even at a concentration of 0.5 mM CHL, floating cells exhibited morphological hallmarks of apoptosis without evidence of necrosis, including nuclear condensation, apoptotic bodies, membrane blebbing, and cytoplasmic condensation (Fig. 1C). Furthermore, cell cycle analysis confirmed the presence of a sub- G_1 peak indicative of apoptosis in the attached cell population after treatment with CHL (Fig. 1D). Thus, 8.3% of the cells treated with 0.5 mM CHL were present in a sub- G_1 peak compared with 0% of the cells in the control cultures. In addition, CHL

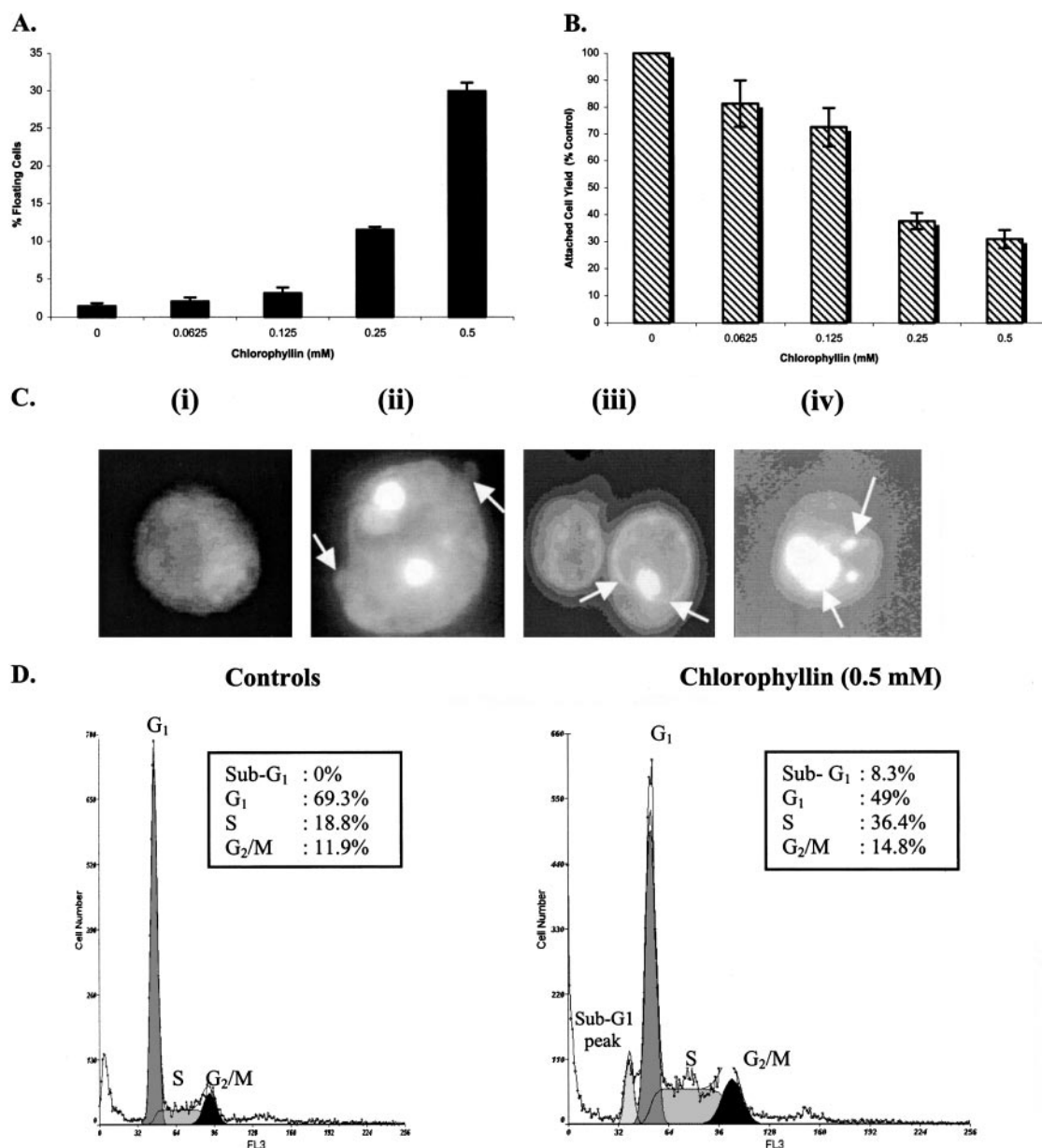


Fig. 1. Induction of apoptosis by CHL in HCT116 human colon cancer cells. *A*, the percentage of floating cells was determined as a measure of the extent of apoptosis induced 24 h after CHL treatment. *B*, the corresponding attached cell yield, calculated as a percentage of the control value (cells not treated with CHL). Results in *A* and *B* are given as mean \pm SD of triplicates and are representative data from three separate experiments. Differences between CHL-treated cells and corresponding controls were significantly different for all CHL concentrations ($P < 0.05$). *C*, fluorescence microscopy of acridine orange/ethidium bromide dual-stained floating cells demonstrating apoptotic morphology: (i), nonapoptotic cell; (ii), membrane blebbing; (iii), cytoplasmic condensation; and (iv), chromatin condensation. *D*, FACS analysis showing the presence of a sub- G_1 peak in the adherent cell population 24 h after CHL treatment. The sub- G_1 peak was confirmed to represent apoptotic cells by examination of ethanol-permeabilized cells containing propidium iodide. MultiCycle software analysis also revealed a decrease in the proportion of cells in G_0 - G_1 and an increase in the DNA content at the S phase of the cell cycle (inset boxes).

treatment caused a decrease in the proportion of cells in G₀-G₁, from 69.3% to 49.0%, and an increase in the fraction of cells in the S-phase of the cell cycle, from 18.8% to 36.4% (Fig. 1D). We are now studying the dose-response relationship between the induction of apoptosis and the accumulation of cells in the S phase of the cell cycle.

CHL Causes Attenuation of the Mitochondrial Membrane Potential ($\Delta\psi_m$) and Release of AIF, without Loss of Cytochrome *c*. To clarify the mechanism(s) of apoptosis by CHL, initial experiments focused on the cytochrome *c*/caspase-3 pathway and downstream targets that might account for morphological features shown in Fig. 1C. Flow cytometry studies revealed a concentration-dependent decrease in $\Delta\psi_m$ after CHL treatment, using Rho-123 as a marker of mitochondrial membrane integrity (Fig. 2A). Because these changes occurred without quenching of the fluorescence signal by CHL and were recapitulated using an alternative marker of $\Delta\psi_m$, JC-1 (data not shown), we next examined likely candidates that might be released from mitochondria. Cytochrome *c* was present in mitochondria at all concentrations of CHL, but surprisingly, no cytochrome *c* was detected in the cytosol, except in positive controls treated with butyrate (Fig. 2B). In contrast, AIF, which is normally localized within the mitochondria, was elevated in both the cytosol and nucleus at all concentrations of CHL (Fig. 2C).

The release of AIF from mitochondria without any detectable increase in cytosolic cytochrome *c* was unexpected and suggested a detailed study of caspases normally regulated by cytochrome *c*/Apaf-1 as well as those activated via alternative mechanisms. A major target of Apaf-1/cytochrome *c* is procaspase-9, which is cleaved to generate

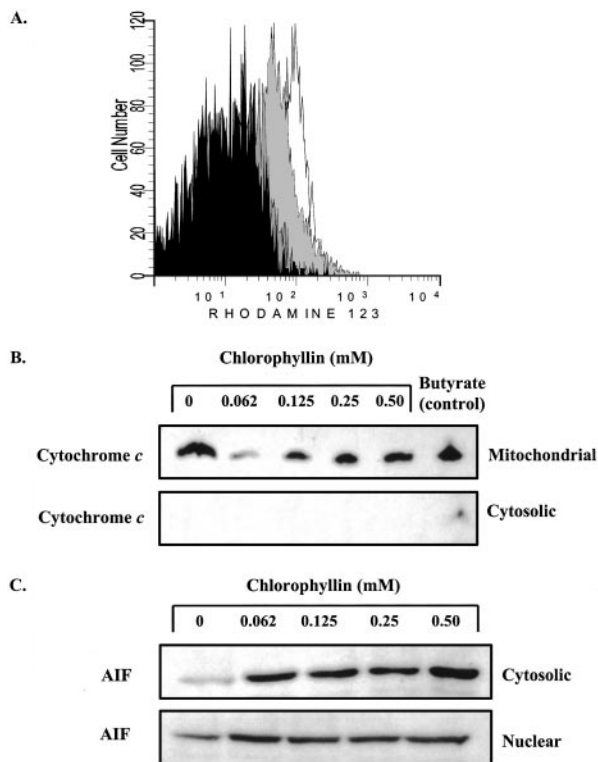


Fig. 2. CHL-induced attenuation of mitochondrial membrane potential ($\Delta\psi_m$) and release of AIF, without loss of cytochrome *c*. *A*, mitochondrial membrane potential $\Delta\psi_m$ was determined in HCT116 cells 6 h after CHL treatment by flow cytometry using Rho-123. Each histogram was shaded according to the corresponding CHL concentration: 0 mM CHL, white; 0.125 mM CHL, gray; 0.25 mM CHL, dark gray; and 0.5 mM CHL, black. Results are representative of findings from three separate experiments. *B*, Western blots of cytochrome *c* expression in mitochondrial and cytosolic fractions obtained from HCT116 cells 24 h after CHL treatment. *C*, Western blots of AIF expression in cytosolic and nuclear fractions 24 h after HCT116 cells were treated with CHL.

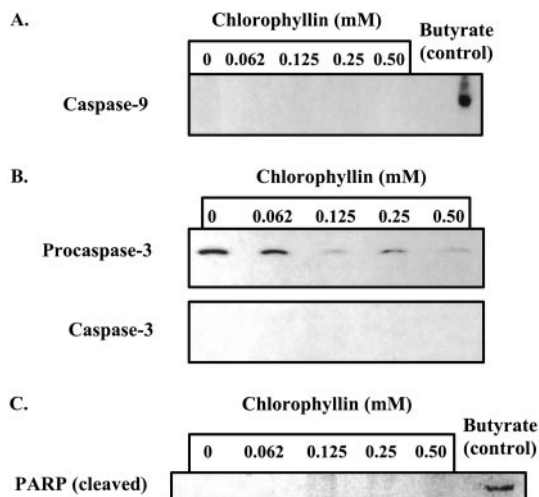


Fig. 3. CHL-induced apoptosis in HCT116 cells does not involve activation of caspase-9, caspase-3, or cleavage of PARP. *A*, Western blot showing the expression of the active cleavage product of procaspase-9 in cells treated with sodium butyrate and the absence of caspase-9 in cells treated with CHL. *B*, expression of procaspase-3 in cells treated with CHL and the absence of caspase-3, the active cleavage product. *C*, immunoblot showing the absence of cleaved PARP in cells treated with CHL. Blots shown in *A*–*C* were from cells examined 24 h after treatment with CHL or butyrate and are representative of results from two or more separate experiments.

caspase-9; the latter form was readily detected in controls treated with sodium butyrate, but not in cells treated with CHL (Fig. 3A). Treatment of HCT116 cells with CHL produced an apparent decrease in the expression of procaspase-3, but no caspase-3 active form was detected (Fig. 3B). Moreover, a major downstream target of caspase-3, namely, PARP, was cleaved in response to treatment with sodium butyrate but not CHL (Fig. 3C).

CHL Induces the Activation of Caspase-8 and Caspase-6, with Cleavage of Nuclear Lamins. Because the mechanism of apoptosis induced by CHL in HCT116 cells apparently did not involve the cytochrome *c*→caspase-9→caspase-3→PARP pathway, additional caspases were examined, including those implicated in membrane “death receptor” pathways. All concentrations of CHL produced the active, cleaved form of caspase-8 (Fig. 4A), and there was a corresponding increase in the active form of caspase-6 at higher concentrations of CHL (Fig. 4B). Lamin A, a major target of caspase-6 (25), was cleaved in a concentration-dependent manner by CHL, and cleaved lamin A products of 45 and 40 kDa were detected in all treatment groups, as well as in the negative and positive controls (Fig. 4C). Indeed, in the latter case, there was extensive accumulation of lamin A cleavage products (Fig. 4C) and oligonucleosomal fragmentation (Fig. 4D), in marked contrast to the results obtained with CHL, in which no DNA laddering was detected under the assay conditions used here. These results clearly establish that the mechanisms by which CHL and butyrate induce apoptosis in HCT116 cells are quite distinct, despite morphological similarities in the floating cell population (Fig. 1; Refs. 13, 23, and 26).

CHL-induced Apoptosis Involves the Cleavage of Bid to Form tBid and Additional Changes in Bcl-2 Family Members. Previous studies showed that caspase-8 cleaves Bid to form tBid, which, through direct association with antiapoptotic members of the Bcl-2 family, releases proapoptotic Bax or Bak to generate pores in the mitochondrial membrane, leading to activation of the cytochrome *c*→caspase-9→caspase-3→PARP pathway (27, 28). In addition, tBid may homodimerize as an alternative mechanism for inducing mitochondrial dysfunction and apoptosis (29, 30). Interestingly, tBid was detected at the higher concentrations of CHL in the present study (Fig. 5A). We therefore examined additional members of the Bcl-2 family

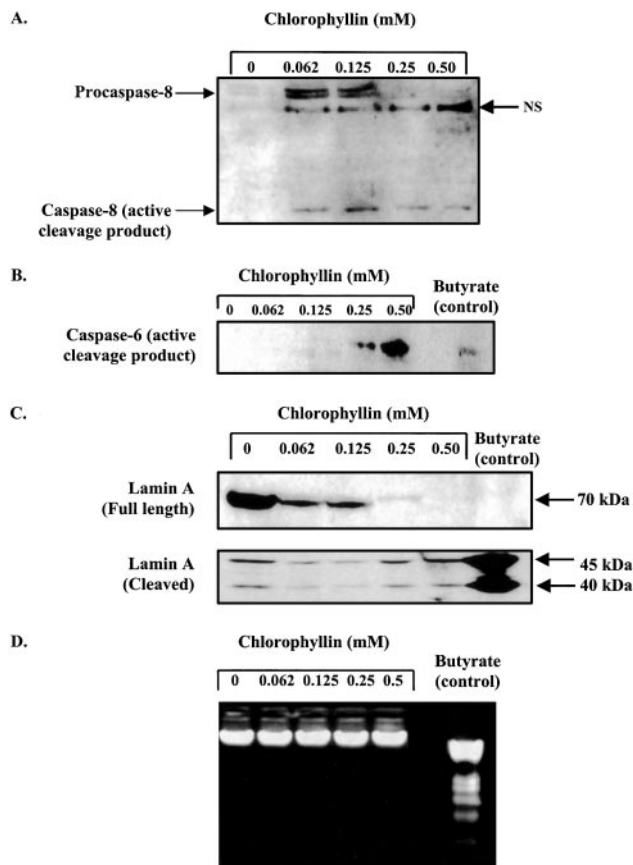


Fig. 4. CHL-induced apoptosis in HCT116 cells involves the activation of procaspase-8 and procaspase-6, with cleavage of nuclear lamins. *A*, Western blot showing the presence of cleaved caspase-8 (active form) at all concentrations of CHL. *NS*, nonspecific band (putative cleavage product). *B*, immunoblot showing the presence of cleaved caspase-6 at higher concentrations of CHL. *C*, concentration-dependent cleavage of lamin A by CHL and the presence of cleaved lamin A fragments. *D*, ethidium bromide-stained agarose gel showing a lack of oligonucleosomal DNA fragmentation in the floating cell population collected after treatment with CHL. The positive control for DNA laddering was 4 mM sodium butyrate (*far right lane*). Results in *A–D* are representative of data obtained from three or more separate experiments, 24 h after cells had been treated with CHL (or butyrate).

that might interact with tBid and modulate its pore-forming activity in the mitochondrial membrane. The prototypical antiapoptotic protein Bcl-2 was decreased in a concentration-dependent manner by CHL (Fig. 5*B*). There was a marked increase in the expression of both antiapoptotic Bcl-x_L and proapoptotic Bcl-x_S, and the ratio of these two proteins remained approximately constant with each concentration of CHL treatment. Expression of Bax, the prototypical proapoptotic protein partner of Bcl-2, was reduced by CHL, but there was a marked increase in the expression of the proapoptotic protein Bak [Fig. 5*B*, (*v*)]. Indeed, expression of the latter protein most closely paralleled the changes observed in cell growth characteristics of HCT116 cells after CHL treatment (Fig. 1).

Cell Differentiation Is Induced by Concentrations of CHL That Induce Apoptosis. Cell differentiation is a major determinant of invasion and metastasis because poorly differentiated cancer cells may avoid host defenses. Therefore, we examined the expression of E-cadherin, a marker of colonic cell differentiation, in response to differentiating agents such as vitamin D (24) and butyrate (31). In the present investigation, there was a marked 5–7-fold induction of E-cadherin protein expression 24 h after CHL treatment (Fig. 6*A*), and at the same time, the expression of E-cadherin mRNA was elevated (Fig. 6*B*). A second indicator of differentiation in human colon cancer cells, ALP, also was induced 3–5-fold at the mRNA level 24 h after

CHL treatment (Fig. 6*C*). These results strongly suggest the involvement of cell differentiation as an integral component of the mechanism by which CHL induces apoptosis in HCT116 cells.

DISCUSSION

CHL has been used in a number of clinical situations, including as an oral deodorant (32), and current Food and Drug Administration guidelines allow for ingestion of three 100 mg tablets per day. These doses of CHL have proven effective in a preliminary intervention study against aflatoxin B₁ exposure in a high-risk population (33), but little is known about the actual concentrations achieved *in situ*, either systemically or in the gastrointestinal tract after an oral dose. This is also true in the case of chlorophyll(in) supplements sold in health food stores across the United States, where much higher doses of CHL may be ingested. Previous studies showed no toxic consequences from lifetime exposure of rats to CHL concentrations as high as 1% and 3% of the diet, in which plasma CHL concentrations on the order of 56–116 μg/ml were reported (34). Although the analysis of specific chlorin metabolites was not determined, the reported plasma concentrations would correspond approximately to 80–160 μM CHL. Concentrations of 62.5 and 125 μM CHL in the present study caused a reduction in the attached cell yield (Fig. 1) and marked changes in several of the molecular markers examined, including increased cytosolic and nuclear AIF levels (Fig. 2*C*), activation of caspase-8 (Fig. 4*A*), cleavage of lamin A (Fig. 4*C*), increased expression of Bak (Fig. 5*B*), and induction of E-cadherin and ALP (Fig. 6). Thus, many of the molecular and morphological changes that were induced at the highest concentrations of 250 and 500 μM CHL were also found in cells

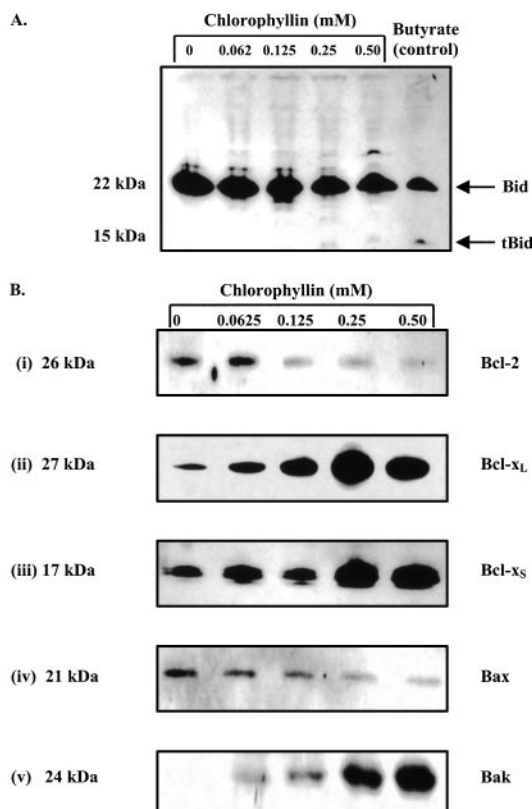


Fig. 5. CHL-induced apoptosis in HCT116 cells involves the cleavage of Bid to form tBid and other changes in pro- and antiapoptotic Bcl-2 family members. *A*, immunoblot showing the presence of tBid in cells treated with 0.25 and 0.5 mM CHL. *B*, immunoblots showing reduced expression of Bcl-2 and Bax proteins and increased levels of Bcl-x_L, Bcl-x_S, and Bak. Results in *A* and *B* are representative of data obtained from two or more separate experiments, 24 h after cells had been treated with CHL (or butyrate).

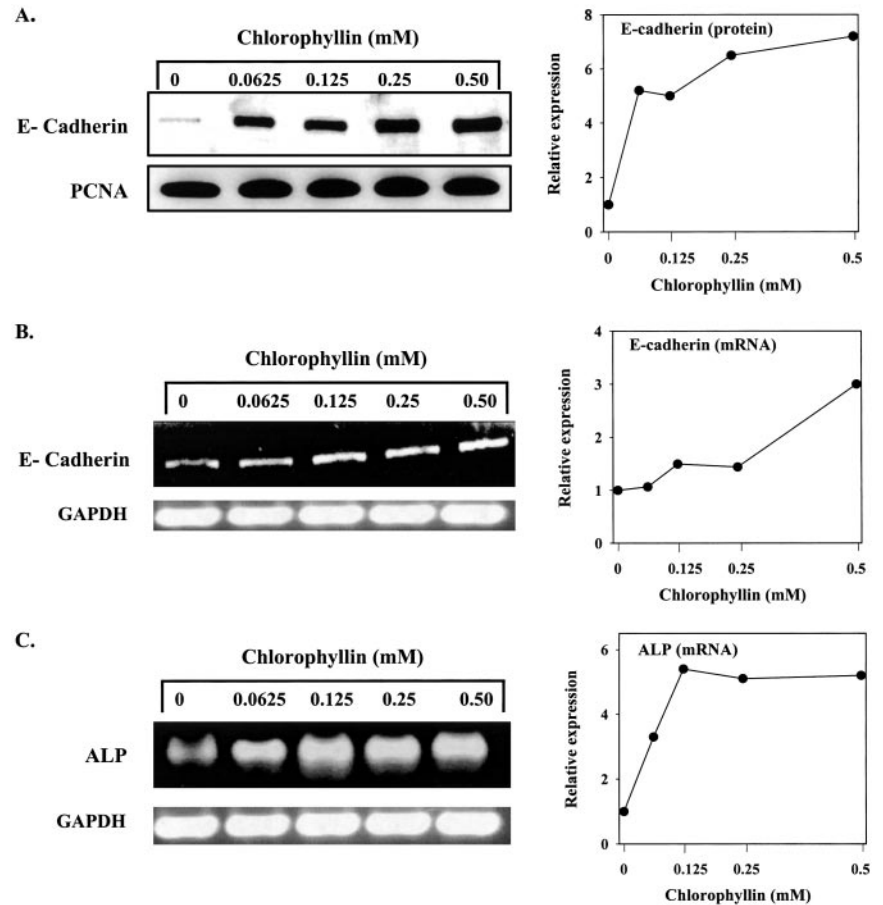


Fig. 6. CHL-induced cell differentiation in HCT116 cells, as evidenced by increased expression of E-cadherin and ALP. *A*, immunoblot showing marked increases in E-cadherin expression 24 h after exposure to CHL. The loading control was PCNA. *B* and *C*, ethidium bromide-stained agarose gels of products obtained by RT-PCR, showing (respectively) increased expression of E-cadherin and ALP mRNA in response to CHL treatment. The housekeeping gene *GAPDH* was used as a control. Results are representative of data obtained from two or more separate experiments. Semiquantitative analysis of immunoblots and gels was performed using an AlphaImnotech gel documentation system and associated software.

treated at lower concentrations, within the range that might reasonably be anticipated to occur *in situ*. Importantly, several of the key molecular markers of apoptosis altered by CHL treatment also responded to purified chlorins, such as chlorin- e_6 , with corresponding changes in cell growth characteristics and apoptosis.⁴

The present study has shown that in HCT116 cells treated with CHL, there was a marked increase in the proportion of floating cells, and that these cells exhibited morphological hallmarks of apoptosis, including nuclear condensation and formation of a sub- G_1 peak during FACS analysis (Fig. 1). Although CHL treatment produced changes in the mitochondrial membrane potential ($\Delta\psi_m$) and release of AIF, no cytochrome *c* was detected in the cytosolic fraction (Fig. 2), and downstream players such as caspase-9, caspase-3, and PARP were not affected, in marked contrast to the results obtained with butyrate (Fig. 3). Interestingly, in a recent study, tumor necrosis factor α -induced cell death in HeLa cells was augmented by rottlerin through a cytochrome *c*-independent pathway, and this involved release of mitochondrial AIF into the cytosol (35). In addition, rottlerin enhanced the activation of caspase-8 and cleavage of Bid (35), as found in the present studies with CHL (Figs. 4 and 5).

Thus, it is possible that the major route for CHL-induced apoptosis involves caspase-8-mediated destruction of nuclear lamins via caspase-6, with concomitant cleavage of Bid to form tBid, the release of AIF from mitochondria, and additional elimination of nuclear lamins (36). Although tBid can interact with antiapoptotic Bcl-2 proteins and displace Bax, initiating pore formation in mitochondria (37), the present study has shown that Bax was markedly reduced by CHL treatment (Fig. 5*B*). The reduced expression of Bax might

explain, in part, the apparent lack of cytochrome *c* release from mitochondria in response to CHL treatment. However, tBid has also been reported to induce the oligomerization of proapoptotic Bak, as well as form homo-oligomers, resulting in cytochrome *c* release from mitochondria (30). The fact that tBid and Bak were increased in response to CHL treatment raises the question as to why AIF but not cytochrome *c* was released from mitochondria. Clearly, the involvement of specific Bcl-2 family members in the apoptotic mechanism and the nature of the $\Delta\psi_m$ transition remain to be clarified, and we are investigating the time course for tBid formation and AIF appearance in the cytosol and nucleus.

An important observation from the present study was the finding that CHL increased the expression of E-cadherin and ALP, which are markers of cell differentiation. In tissues undergoing continuous regeneration, such as the colon, homeostatic mechanisms seek to balance the birth of cells in the lower half of the crypt with programmed cell death in the upper half of the crypt. Dysregulation of this homeostatic balance, via a relative increase in the rate of cell proliferation or a decrease in the rate of apoptosis, provides a mechanism for tumorigenesis, and many chemopreventive agents in the colon act by enhancing apoptosis (12, 23, 31). However, at later stages, it is the differentiation status of the cancer that frequently dictates the response to chemopreventive and chemotherapeutic agents (13, 15, 24). The present study indicates, for the first time, that human colon carcinoma cells respond to CHL treatment by undergoing differentiation and that this occurred at all concentrations in the range 62.5–125 μM CHL. Additional work is now in progress to define the low-dose threshold for induction of cell differentiation by CHL and determine whether such changes occur before the induction of apoptosis. However, the results presented here for E-cadherin and ALP suggest the

⁴ G. D. Díaz and R. H. Dashwood, unpublished results.

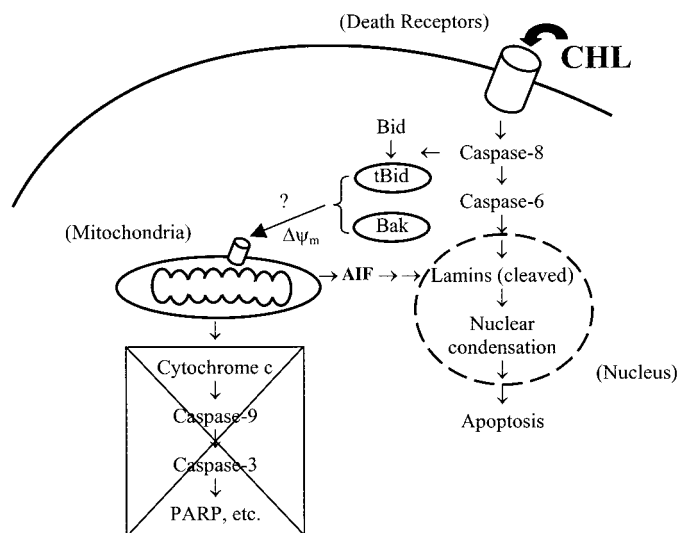


Fig. 7. Model for the mechanism of apoptosis induced by CHL in HCT116 cells. Activation of death receptors by CHL results in the initial cleavage of procaspase-8 to generate caspase-8. Caspase-8 represents a branch point, activating caspase-6 in one direction and cleaving Bid to form tBid in another direction. Increased levels of tBid and other proapoptotic Bcl-2 family members (e.g., Bak) facilitate a change in mitochondrial membrane potential ($\Delta\psi_m$), leading to the release of AIF (but not cytochrome *c*). As a consequence, nuclear lamins are cleaved, leading to nuclear condensation and apoptosis.

possibility that, in addition to its role as a chemopreventive agent, CHL might also have important clinical uses as a cancer therapeutic agent. It has been noted previously that CHL is a highly attractive compound in the chemoprevention setting because it lacks toxicity and is widely available and affordable—indeed, CHL may be effective for only “pennies a day” (32). The present investigation suggests that additional studies are warranted on the potential use of CHL as a cancer therapeutic agent in the colon, acting via induction of cell differentiation.

Finally, an interesting feature of the apoptotic mechanism induced by CHL after 24 h was the apparent lack of oligonucleosomal DNA fragmentation, in contrast to the results obtained with butyrate (Fig. 4D). This finding indicates that under certain circumstances, HCT116 cells can detach and undergo many of the morphological changes characteristic of apoptosis, without evidence of DNA laddering. It is possible that DNA fragmentation might be detected with prolonged CHL treatment, as in prior studies *in vivo* that identified an increase in the colonic crypt terminal deoxynucleotidyl transferase-mediated nick end labeling index in rats after 16 weeks of CHL exposure (12). However, it is also possible that the apoptotic mechanisms induced by CHL in normal cells differ from the mechanisms induced in colon cancers, due, for example, to the nature of key death receptors in cancer cells. In the latter situation, activation of nuclear condensation without extensive nuclear DNA fragmentation may suffice to trigger the elimination of carcinomas from the colon. A major focus of current investigation is to identify the putative death receptors for CHL on colon cancer cells, as well as establishing possible cross-talk with apoptotic pathways involving death receptors such as Fas/CD95, TNFR1, DR3/APO-3, and DR4/5 (37–39).

In summary, CHL was shown for the first time to induce apoptosis in HCT116 human colon cancer cells, and this was accompanied by a marked increase in cell differentiation. Based on the results obtained to date, our working hypothesis (Fig. 7) is that the cytochrome *c*→caspase-9→caspase-3→PARP pathway is unlikely to be involved in the mechanism of CHL-induced apoptosis but that the interaction of CHL with putative death receptors activates the caspase-8→caspase-6→nuclear lamin pathway, resulting in nuclear condensation. Activa-

tion of caspase-8 also leads to the formation of tBid, which, in conjunction with changes in Bak and other Bcl-2 family members, facilitates the release of AIF from mitochondria via a $\Delta\psi_m$ transition that does not release high levels of cytochrome *c*. Subsequently, AIF enhances the cleavage of nuclear lamins and activates the process of nuclear condensation, although the specific timing and relative contributions of AIF *versus* the caspase-8/caspase-6 pathway remain to be determined.

ACKNOWLEDGMENTS

We thank Professor George S. Bailey and Margaret Pratt of the Department of Environmental and Molecular Toxicology (Oregon State University, Corvallis, OR) for high-performance liquid chromatography analyses of CHL samples and characterization of chlorins. We also thank Julie Oughton of the Environmental Health Sciences Center (Oregon State University) for assistance with the FACS analyses.

REFERENCES

- Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* (Wash. DC), 292: 727–730, 2001.
- Desagher, S., and Martinou, J. C. Mitochondria as the central control point of apoptosis. *Trends Cell Biol.*, 10: 369–377, 2000.
- Yoshida, H., Kong, Y.-Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M., and Mak, T. W. Apaf-1 is required for mitochondrial pathways of apoptosis and brain development. *Cell*, 94: 739–750, 1998.
- Woo, M., Hakem, R., Soengas, M. S., Ducan, G. S., Shahinian, A., Kagi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, *et al.* Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev.*, 12: 806–819, 1998.
- Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* (Lond.), 391: 43–50, 1998.
- Sakahira, H., Enari, M., and Nagata, S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* (Lond.), 391: 96–99, 1998.
- Marsden, V. S., O'Connor, L., O'Reilly, L. A., Silke, J., Metcalf, D., Ekert, P. G., Huang, D. C. S., Cecconi, F., Kuida, K., Tomaselli, K. J., Roy, S., Nicholson, D. W., Vaux, D. L., Bouillet, P., Adams, J. M., and Strasser, A. Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome *c*/Apaf-1/caspase-9 apoptosome. *Nature* (Lond.), 419: 634–637, 2002.
- Negishi, T., Rai, H., and Hayatsu, H. Antigenotoxic activity of natural chlorophylls. *Mutat. Res.*, 376: 97–100, 1997.
- Dashwood, R. H., Negishi, T., Hayatsu, H., Breinholt, V., Hendricks, J. D., and Bailey, G. S. Chemopreventive properties of chlorophylls towards aflatoxin B₁: a review of the antimutagenicity and anticarcinogenicity data in rainbow trout. *Mutat. Res.*, 399: 245–253, 1998.
- Dashwood, R. H. Chlorophylls as anticarcinogens. *Int. J. Oncol.*, 10: 721–727, 1997.
- Blum, C. A., Xu, M., Orner, G. A., Fong, Q. T., Bailey, G. S., Stoner, G. D., Horio, D. T., and Dashwood, R. H. β -Catenin mutation in rat colon tumors initiated by 1,2-dimethylhydrazine and 2-amino-3-methylimidazo[4,5-f]quinoline, and the effect of post-initiation treatment with chlorophyllin and indole-3-carbinol. *Carcinogenesis* (Lond.), 22: 315–320, 2001.
- Dashwood, R. H., Xu, M., Orner, G. A., and Horio, D. T. Colonic cell proliferation, apoptosis and aberrant crypt foci development in rats given 2-amino-3-methylimidazo[4,5-f]quinoline. *Eur. J. Cancer Prev.*, 10: 139–145, 2001.
- Hague, A., Díaz, G. D., Hicks, D. J., Krajewski, S., Reed, J. C., and Paraskeva, C. bcl-2 and bak may play a pivotal role in sodium butyrate-induced apoptosis in colonic epithelial cells; however, overexpression of bcl-2 does not protect against bak-mediated apoptosis. *Int. J. Cancer*, 72: 898–905, 1997.
- Elder, D. J., Hague, A., Hicks, D. J., and Paraskeva, C. Differential growth inhibition by the aspirin metabolite salicylate in human colorectal tumor cell lines: enhanced apoptosis in carcinoma and *in vitro*-transformed adenoma relative to adenoma cell lines. *Cancer Res.*, 56: 2273–2276, 1996.
- Díaz, G. D., Paraskeva, C., Thomas, M. G., Binderup, L., and Hague, A. Apoptosis is induced by the active metabolite of vitamin D₃ and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy. *Cancer Res.*, 60: 2304–2312, 2000.
- Wang, Q., Li, N., Wang, X., Kim, M. M., and Evers, B. M. Augmentation of sodium butyrate-induced apoptosis by phosphatidylinositol 3'-kinase inhibition in the KM20 human colon cancer cell line. *Clin. Cancer Res.*, 8: 1940–1947, 2002.
- Ashok, P. R., Hartig, U., Barth, M. C., Baird, W. M., Schimerlik, M., Hendricks, J. D., and Bailey, G. S. Inhibition of dibenzo[*a,l*]pyrene-induced multi-organ carcinogenesis by dietary chlorophyllin in rainbow trout. *Carcinogenesis* (Lond.), 20: 1919–1926, 1999.

18. Egner, P. A., Stanbury, K. H., Snyder, E. P., Rogers, M. E., Hintz, P. A., and Kensler, T. W. Identification and characterization of chlorin e_4 ethyl ester in sera of individuals participating in the Chlorophyllin Chemoprevention Trial. *Chem. Res. Toxicol.*, *13*: 900–906, 2000.
19. Peterson, G. L. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.*, *83*: 346–356, 1977.
20. Huijsloot, M., Tijdens, I. B., Mulder, G. J., and van de Water, B. Differential regulation of doxorubicin-induced mitochondrial dysfunction and apoptosis by Bcl-2 in mammary adenocarcinoma (MTLn3) cells. *J. Biol. Chem.*, *277*: 35869–35879, 2002.
21. Paquin, J., Danalache, B. A., Jankowski, M., McCann, S. M., and Gutkowska, J. Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. *Proc. Natl. Acad. Sci. USA*, *99*: 9550–9555, 2002.
22. Premkumar, A., and Simantov, R. Mitochondrial voltage-dependent anion channel is involved in dopamine-induced apoptosis. *J. Neurochem.*, *82*: 345–352, 2002.
23. Hague, A., Manning, A. M., Hanlon, K. A., Huschtscha, L. I., Hart, D., and Paraskeva, C. Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int. J. Cancer*, *55*: 498–505, 1993.
24. Palmer, H. G., Gonzalez-Sancho, J. M., Espada, J., Berciano, M. T., Puig, I., Baulida, J., Quintanilla, M., Cano, A., de Herreros, A. G., Lafarga, M., and Munoz, A. Vitamin D₃ promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β -catenin signaling. *J. Cell Biol.*, *154*: 369–387, 2001.
25. Goldberg, M., Harel, A., and Gruenbaum, Y. The nuclear lamina: molecular organization and interaction with chromatin. *Crit. Rev. Eukaryotic Gene Expression*, *9*: 285–293, 1999.
26. Archer, S. Y., Meng, S., Shei, A., and Hodin, R. A. p21^{WAF1} is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc. Natl. Acad. Sci. USA*, *95*: 6791–6796, 1998.
27. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. Cytochrome *c* and dATP-dependent formation of Apaf-2/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, *91*: 479–489, 1997.
28. Green, D. R., and Reed, J. C. Mitochondria and apoptosis. *Science (Wash. DC)*, *281*: 1309–1312, 1998.
29. Kim, T.-H., Zhao, Y., Barber, M. J., Kurharsky, D. K., and Yin, X.-M. Bid-induced cytochrome *c* release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. *J. Biol. Chem.*, *275*: 39474–39481, 2000.
30. Grinberg, M., Sarig, R., Zaltsman, Y., Frumkin, D., Grammatikakis, N., Reuveny, E., and Gross, E. tBid homooligomerizes in the mitochondrial membrane to induce apoptosis. *J. Biol. Chem.*, *277*: 12237–12245, 2002.
31. Barshishat, M., Polak-Charcon, S., and Schwartz, B. Butyrate regulates E-cadherin transcription, isoform expression and intracellular position in colon cancer cells. *Br. J. Cancer*, *82*: 195–203, 2000.
32. Young, R. W., and Beregi, J. S., Jr. Use of chlorophyllin in the care of geriatric patients. *J. Am. Geriatr. Soc.*, *28*: 46–47, 1980.
33. Egner, P. A., Wang, J. B., Zhu, Y. R., Zhang, B. C., Wu, Y., Zhang, Q. N., Quian, G. S., Kuang, S. Y., Gange, S. J., Jacobson, L. P., Helzlsouer, K. J., Bailey, G. S., Groopman, J. D., and Kensler, T. W. Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. *Proc. Natl. Acad. Sci. USA*, *98*: 14601–14606, 2001.
34. Harrison, J. W. E., Levin, S. E., and Trabin, B. The safety and fate of potassium sodium copper chlorophyllin and other copper compounds. *J. Am. Pharm. Assoc.*, *18*: 722–737, 1954.
35. Basu, A., Johnson, D. E., and Woolard, M. D. Potentiation of tumor necrosis- α -induced cell death by rottlerin through a cytochrome-*c*-independent pathway. *Exp. Cell Res.*, *278*: 209–214, 2002.
36. Daugas, E., Susin, S. A., Zamzami, N., Ferri, K. F., Irinopoulou, T., Larochette, N., Prevost, M. C., Leber, B., Andrews, D., Penninger, J., and Kroemer, G. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J.*, *14*: 729–739, 2000.
37. Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B., and Korsmeyer, S. J. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome *c*. *Genes Dev.*, *14*: 2060–2071, 2000.
38. Ashkenazi, A., and Dixit, V. M. Death receptors: signaling and modulation. *Science (Wash. DC)*, *281*: 1305–1308, 1998.
39. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. Bid, a Bcl-2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. *Cell*, *94*: 481–490, 1998.

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Caspase-8 and Apoptosis-inducing Factor Mediate a Cytochrome *c*-independent Pathway of Apoptosis in Human Colon Cancer Cells Induced by the Dietary Phytochemical Chlorophyllin

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Cancer Res 2003;63:1254-1261.

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