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Effects of 5-Azacytidine and Butyrate on Differentiation and Apoptosis of Hepatic Cancer Cell Lines

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Objective

To determine the cellular effects of 5-azacytidine (5-azaC) and sodium butyrate on two human liver cancers, HepG2 and Hep3B.

Summary Background Data

Primary liver cancer is a significant health problem; treatment options are limited and prognosis is poor. Recent studies have focused on the role that programmed cell death (*i.e.*, apoptosis) plays in both normal and neoplastic growth: certain genes can either suppress (*e.g.*, Bcl-2, Bcl-xL) or promote (*e.g.*, Bik, Bax, Bak) apoptosis. The identification of novel agents targeted to specific molecular pathways may be beneficial in the treatment of this disease.

Methods

Human liver cancer cell lines HepG2 and Hep3B were treated with 5-azaC alone, butyrate alone, or 5-azaC and butyrate. Morphologic and proliferative changes were assessed by light microscopy and 5-bromo-2′-deoxyuridine staining; flow cytometry was used to determine cell cycle characteristics. Apoptosis was assessed by DNA laddering and the *in situ* apoptosis detection assay using the TdT-mediated dUTP nick end labeling method. In addition, total RNA and protein were analyzed by ribonuclease protection and Western blot, respectively, to assess changes in the expression of apoptosis-related genes.

Results

Treatment with either 5-azaC or butyrate inhibited cell growth and induced apoptosis in both HepG2 and Hep3B cells; the combination of 5-azaC and butyrate was not more effective than either agent alone. 5-azaC alone resulted in a more differentiated-appearing morphology and G_2 cell cycle arrest in both cell lines. Treatment with 5-azaC or butyrate affected the expression levels of proteins of the Bcl-2 family.

Conclusions

Both 5-azaC and butyrate induced apoptosis in the HepG2 and Hep3B liver cancer cells; 5-azaC treatment alone produced G_2 arrest in both cell lines. Proteins of the Bcl-2 family may play a role in the cellular changes that occur with treatment, but further studies are required to define this potential role. Products of the apoptotic pathway may prove to be useful therapeutic targets in the treatment of hepatic cancers.

Primary liver cancers remain one of the most common malignancies worldwide, with an annual incidence of approximately 1 million cases.[1,2] The global differences in the incidence of hepatic cancer (particularly hepatocellular cancer, which is the most common primary liver cancer) are dramatic, with the highest incidence (approximately 730 cases per 100,000 males/year) found in sub-Saharan Africa, Southeast Asia, and southern China.[2–5] In contrast, northern Europe and North America have annual rates of less than two cases per 100,000 males. Several potential etiologic causes have been described for hepatocellular cancer, including a strong association with the hepatitis virus, alcoholic cirrhosis, and several environmental toxins.[2–4] Surgical resection remains the mainstay of therapy; however, metastases are often present when the cancer is discovered, precluding a curative resection. Chemotherapy adds little to

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overall survival in patients in whom a curative resection is impossible.[6] Therefore, novel strategies and agents that target specific molecular pathways, as well as triggering a process of cell death (*i.e.*, apoptosis), are being evaluated in the treatment of several neoplasms.

Apoptosis, a normal cellular process that provides for the orderly senescence and death of cells in a programmed fashion, may be positively or negatively modulated by several external or internal factors.[7,8] The molecular factors that contribute to this process are under intense investigation. Apoptosis is distinct from cellular necrosis and can be characterized by morphologic (*i.e.*, cell shrinkage, chromatin condensation, and production of membrane-enclosed particles known as apoptotic bodies) and biochemical (*i.e.*, DNA fragmentation) criteria.[7] Recently, several proteins that are structurally related to Bcl-2, an inhibitor of apoptosis, have been identified.[8] For example, the homologous Bcl-2 and Bcl-xL proteins can extend cell survival by suppressing apoptosis,[9,10] whereas the proapoptotic proteins (*e.g.*, Bax, Bak, Bik) act as dominant cell death inducers when overexpressed.[10]

Apoptosis also plays an important role in cancers, as noted by a dysregulation of the normal cell death process in several neoplasms.[11] In recent years, strategies aimed at modulating the apoptotic pathway have been described as possible treatments for different cancers. For example, the cytidine analog 5-azacytidine (5-azaC) incorporates into the genomic DNA, resulting in hypomethylation of genes with differentiation or apoptosis in certain cells.[12–16] 5-azaC was developed as a potent anticancer agent for the treatment of myeloid leukemia and has been shown to induce apoptosis in the human promyelocytic leukemia cell line HL-60.[17–19] The short-chain fatty acid sodium butyrate is another well-characterized differentiating agent that induces growth arrest and differentiation in several malignant cell lines.[20,21] In addition, butyrate promotes apoptosis *in vitro* in leukemia cells and human colon, breast, and lung cancer cell lines. The effects of 5-azaC and butyrate on human liver cancers are largely undefined.

The purpose of our study was to evaluate the effects of 5-azaC and butyrate on differentiation and apoptosis of two human liver cancer cell lines, Hep3B, a hepatoma line, and HepG2, a hepatoblastoma line. Morphologic and proliferative changes, cell cycle characteristics, and biochemical assessments were determined after treating cells with these agents. In addition, an initial evaluation of potential molecular mediators for the effects of 5-azaC and butyrate was performed.

MATERIALS AND METHODS

Cell Culture

HepG2 and Hep3B, human liver cancer cell lines[22,23] obtained from the American Type Culture Collection (Rockville, MD), were cultured in Eagle's MEM with 10% fetal bovine serum and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For all experiments, cells were treated with 5-azaC (8 μM added 24 hours after plating), sodium butyrate (35 mM added 24 hours before harvesting), or both. Cells were harvested 4 days after 5-azaC treatment when morphologic changes were present. Control cells were treated with the vehicle (phosphate-buffered saline [PBS]) used to dilute 5-azaC and butyrate.

5-Bromo-2′-Deoxyuridine Immunocytochemical Analysis

For assessment of cell proliferation, 5-bromo-2′-deoxyuridine (BrdU) immunocytochemistry was performed with a Cell Proliferation Kit (Amersham, Arlington Heights, IL), as described by the manufacturer. Briefly, cells were incubated with labeling medium containing BrdU and 5-fluoro-2′-deoxyuridine at 37°C for 1 hour. Cells were fixed in 10% buffered formalin for 30 minutes and washed with PBS, followed by incubation with reconstituted nuclease/anti-BrdU antibody for 1 hour. After washing with PBS, the samples were incubated with peroxidase antimouse IgG_{2a} for 30 minutes, washed with PBS, stained with 3,3′-diaminobenzidine tetrahydrochloride solution, and visualized by light microscopy.

DNA Fragmentation Assay

DNA was extracted from control and treated cells (including attached and floating cells) using an apoptotic DNA ladder kit (Boehringer Mannheim, Indianapolis, IN), according to the manufacturer's directions. Briefly, cells were pelleted and resuspended in 200 μl of PBS, mixed with 200 μl of binding buffer, and then incubated for 10 minutes at 72°C. The samples were mixed with 100 μl isopropanol, centrifuged, and washed two additional times with wash buffer. The DNA was eluted with the elution buffer, resolved by electrophoresis on a 2% agarose gel, and visualized by ethidium bromide staining.

In Situ Apoptosis Detection

Apoptosis was further confirmed by the TdT-mediated dUTP nick end labeling (TUNEL) method using a detection kit from Boehringer Mannheim. Briefly, cells were fixed in a 4% paraformaldehyde solution (pH 7.4) for 30 minutes, washed with PBS, and incubated with 0.3% H_2O_2 in methanol for 30 minutes. Cells were then washed with PBS, incubated in permeabilization solution (0.1% Triton X-100 in 0.1 sodium citrate) for 2 minutes on ice, and incubated with TUNEL reaction mixture at 37°C for 30 minutes. Samples were washed with PBS, incubated with converter-POD at 37°C for 1 hour and stained with 3,3′-diaminobenzidine tetrahydrochloride solution. A negative control using all reagents except terminal transferase was performed in

parallel. The nuclei of positive (*i.e.*, apoptotic) cells stained brown as detected by light microscopy.

Cell Cycle Analysis

The effects of 5-azaC and butyrate on the cell cycle progression of HepG2 and Hep3B cells were analyzed by fluorocytometry. Briefly, cells were collected in ice-cold PBS and incubated with ribonuclease (RNase; 100 μg/ml) for 30 minutes at 37°C. Cells were stained with a propidium iodine solution (50 μg/ml; Sigma, St. Louis, MO) for 15 minutes. Cell cycle analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and cell cycle distribution was analyzed by the Modfit LT program (Verity, ME).

Probe Preparation, RNA Extraction, and RNase Protection Assay

A multiprobe template (hApo-2) for the analysis of the Bcl-2 gene family was obtained from PharMingen (San Diego, CA). ^{32}P-labeled antisense RNA was transcribed using T7 RNA polymerase (Promega, Madison, WI) and [α-^{32}P]CTP (Dupont-New England Nuclear, Boston, MA). Total RNA was extracted from cells using the Ultraspec II (Biotecx Laboratories, Houston, TX) RNA isolation system, according to the manufacturer's conditions.[24] RNase protection assays were performed using the RPA-II kit from Ambion (Austin, TX). Briefly, total RNA (50 μg) was hybridized with the ^{32}P-labeled hApo-2 multiprobe overnight at 45°C, followed by digestion with a 1:250 dilution of Solution R (RNase A/RNase T1 mixture) for 30 minutes at 37°C. RNA pellets were dried briefly and resuspended in 8 μl of a formamide loading buffer, and RNase-resistant fragments were separated on a 5% polyacrylamide–8 M urea gel and visualized by autoradiography.

Protein Preparation, Immunoprecipitation, and Western Blot

Cells were collected by scraping and centrifugation (2000 g for 10 minutes). Cell pellets were lysed with TNN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine hydrochloride, and 25 μg/ml of aprotinin) for 30 minutes at 4°C. Lysates were clarified by centrifugation at 10,000 g for 30 minutes at 4°C, and protein concentrations were determined using the method of Bradford.[25]

Protein samples (250 μg) were then immunoprecipitated with primary antibody (all antibodies were from Santa Cruz) for 1 hour and incubated with protein G beads overnight at 4°C with constant rotation. After centrifugation, the beads were washed three times with PBS and TNN buffer and assayed as described for Western blots. Western blot analysis was performed as described previously.[26] Briefly, protein samples were resolved by 10% SDS-polyacrylamide gel electrophoresis and electroblotted to Immobilon-P nylon membranes. Filters were incubated overnight at 4°C in blocking solution (Tris-buffered saline containing 5% nonfat dried milk and 0.05% Tween 20), followed by a 3-hour incubation with primary antibody (1:200 dilution). Filters were washed three times and incubated with a peroxide-conjugated antibody (1:1000 to 1:5000 dilution) for 1 hour. After four final washes, the immune complexes were visualized using ECL detection.

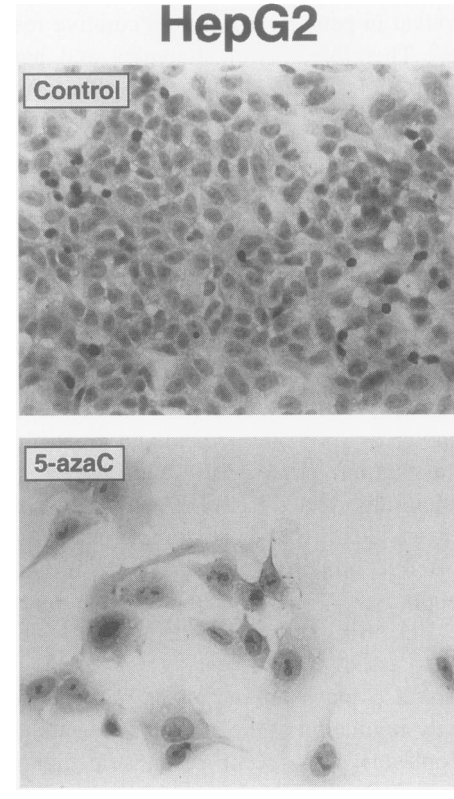

Figure 1. Assessment of morphologic changes in HepG2 after treatment with 5-azaC. HepG2 cells treated with 5-azaC or vehicle (control) were assessed by light microscopy (×200). HepG2 cells treated with 5-azaC exhibited a more differentiated-appearing morphology than control cells.

RESULTS

Treatment With 5-azaC and Butyrate Promoted Apoptosis of Both Liver Cancer Cell Lines

We first assessed the effects of 5-azaC and butyrate, either alone or in combination, on the morphology and proliferation of HepG2 and Hep3B using BrdU immunocytochemistry and light microscopy. Treatment with 5-azaC produced marked morphologic changes in both HepG2 and Hep3B, with the effects more pronounced in HepG2 (Fig. 1). Dramatic increases in cell size and the nuclear/cytoplasmic ratio were noted, with characteristics consistent with a more differentiated

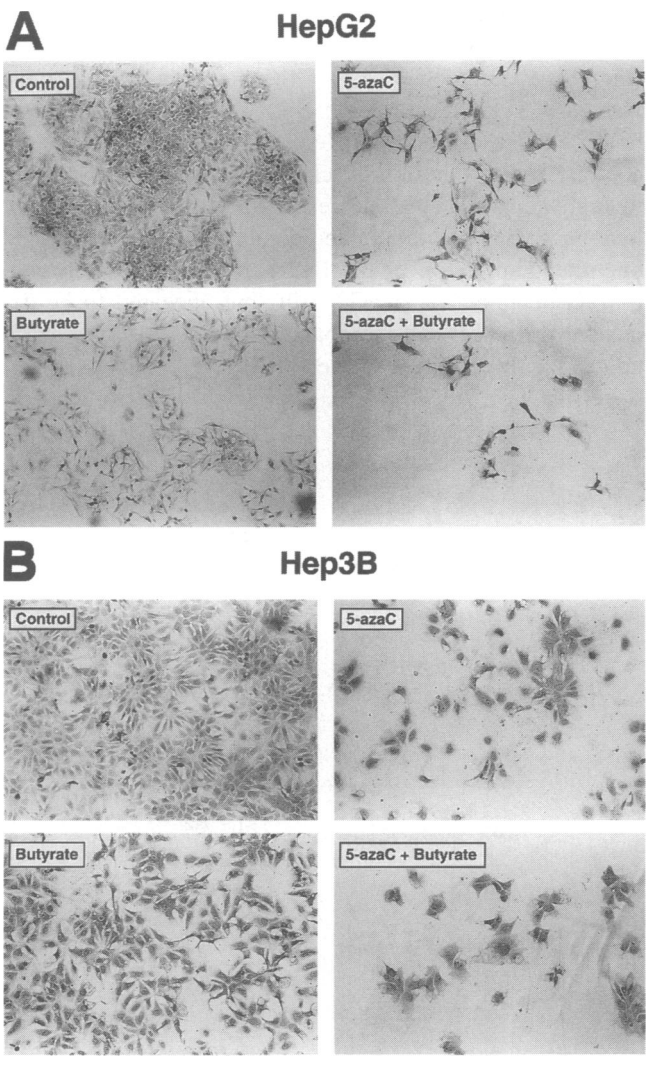

Figure 2. Assessment of proliferation by BrdU immunocytochemistry. HepG2 (A) and Hep3B (B) cells were treated with 5-azaC, sodium butyrate, 5-azaC and butyrate, or vehicle (control) and assessed by BrdU staining and light microscopy (×140). Control HepG2 and Hep3B cells exhibited more nuclei stained positive for BrdU.

morphology. In addition, the 5-azaC-treated Hep3B cells showed more multinucleated cells, suggesting that mitosis was blocked. A decrease in BrdU-stained nuclei was evident in 5-azaC-treated HepG2 and Hep3B cells (Fig. 2). In contrast, treatment with butyrate did not produce the changes in the cellular morphology of either HepG2 or Hep3B that were noted with 5-azaC. Cells did, however, become detached if butyrate treatment was prolonged.

To determine whether the inhibition of cell growth by 5-azaC and butyrate was the result of apoptosis, several techniques were used. A specific biochemical characteristic of apoptosis involves fragmentation of the cellular DNA into discrete oligonucleotides of varying sizes (50 to 300 kb), resulting in a DNA ladder appearance when analyzed by gel electrophoresis.[27] The discontinuous DNA degradation occurring during apoptosis was noted in HepG2 cells treated with either 5-azaC or butyrate (Fig. 3); cells treated with both agents were similar to single-agent treatment alone. DNA laddering was also identified in Hep3B cells treated with either 5-azaC or butyrate (data not shown). Furthermore, the presence of excessive DNA breakage *in situ*, detected by labeling free 3′ OH termini with dUTP in the TUNEL reaction, was noted in HepG2 cells treated with 5-azaC (Fig. 4A). Treatment with butyrate did not appear to increase the number of apoptotic cells as much as treatment with 5-azaC, probably because butyrate induced a rapid apoptotic process, leading to the detachment and floating of cells in the media that could not be assessed by this *in situ* technique. The combination of treatments resulted in a labeling similar to that of 5-azaC alone. Similar findings were noted with Hep3B cells, except that more apoptotic cells were present with butyrate treatment than in HepG2 cells (Fig. 4B). Taken together, these results indicate that apoptosis is induced by 5-azaC and butyrate in both HepG2 and Hep3B cells.

Alterations in the Cell Cycle Associated With 5-azaC and Butyrate Treatment

The proportion of cells in the different phases of the cell cycle was next determined by flow cytometry (Fig. 5). Untreated HepG2 and Hep3B cells demonstrated a relatively normal distribution pattern, with most cells in the G_1 phase of the cycle and a lower G_2 peak. Treatment with 5-azaC significantly increased the distribution of cells in the G_2 phase, with a concomitant increase in the proportion of hyperdiploid cells, which suggests G_2 arrest of the cells. HepG2 cells treated with both agents demonstrated an increase in both G_1 and G_2 distribution, with a decrease in the number of hyperdiploid cells compared with 5-azaC alone.

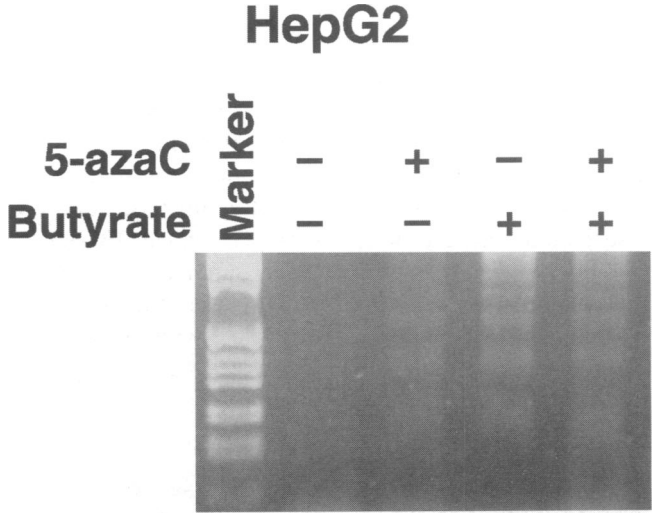

Figure 3. DNA fragmentation assay. Agarose gel electrophoresis of DNA extracted from HepG2 cells treated with 5-azaC or sodium butyrate. The ladder pattern of DNA fragmentation was observed in cells treated with either agent but not in untreated cells. The left lane contains molecular weight markers.

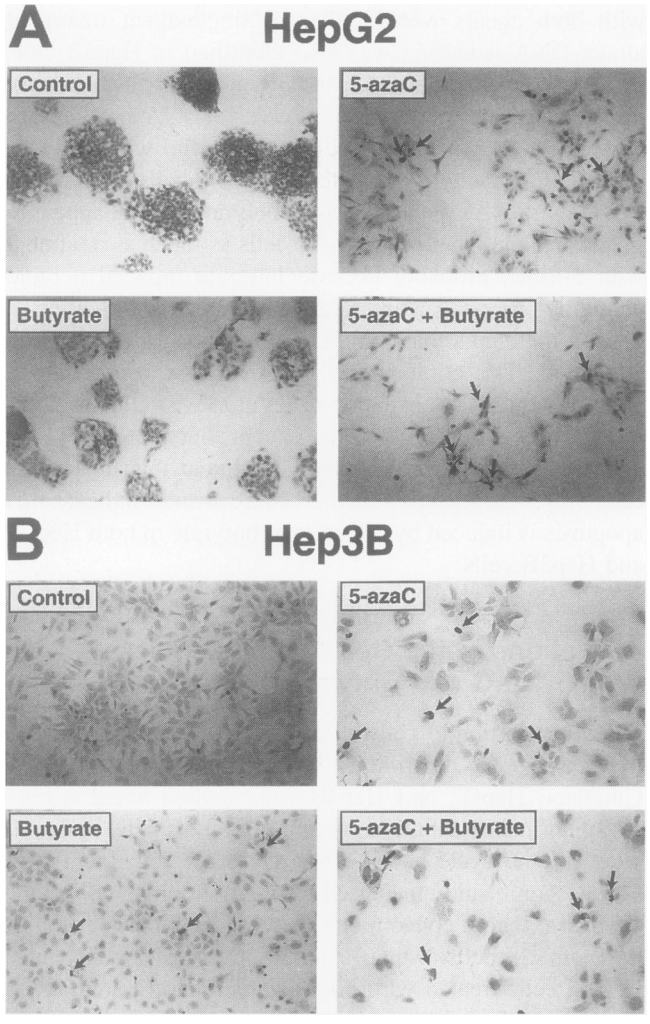

Figure 4. *In situ* apoptosis detection. HepG2 (A) and Hep3B (B) cells treated with 5-azaC, butyrate, 5-azaC and butyrate, or vehicle (control) and analyzed for apoptosis by the TUNEL method. The nuclei of positive (*i.e.*, apoptotic) cells stain brown (arrows) (×140).

Hep3B cells treated with both agents showed a lower G_2 peak and no G_1 peak, but more hyperdiploid cells than with 5-azaC alone. This increase in hyperdiploid cells was consistent with our morphologic observations and further suggest a cell cycle arrest occurring before mitosis. Cell cycle distribution was not altered in HepG2 or Hep3B cells in response to butyrate treatment. In summary, our results indicate arrest of the cells in the G_2 phase of the cell cycle by treatment with 5-azaC and correlate with our previous findings demonstrating changes in cell morphology and proliferation.

Molecular Alterations in Response to Treatment of the Liver Cancer Cell Lines

To begin to elucidate the molecular mechanisms responsible for the cellular changes noted after 5-azaC and butyrate treatment, an RNase protection analysis was performed using a multiprobe capable of assessing changes in expression of members of the Bcl-2 family simultaneously (Fig. 6). Expression levels of the proapoptotic Bik gene were low in untreated cells; Bik expression increased in both cell lines treated with either 5-azaC or butyrate. In contrast, expression of the Bcl-xL gene, which was present in untreated cells, was decreased in both cell lines after treatment with butyrate or the combination of 5-azaC and butyrate. Expression of the Bax gene was increased with 5-azaC treatment in HepG2 cells, but appeared to be decreased in Hep3B cells compared with control. Minimal to no changes were noted in the expression of Bcl-2 or Bak. Levels of L32 and GAPDH, two constitutively expressed

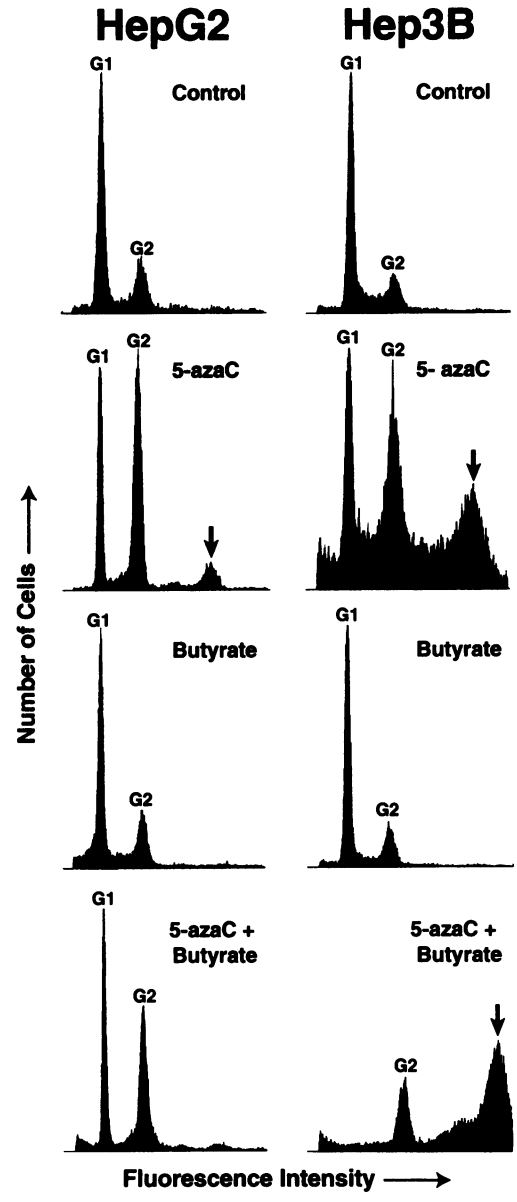

Figure 5. Flow cytometric analysis. DNA content analysis of HepG2 and Hep3B cells treated with 5-azaC, butyrate, 5-azaC and butyrate, or vehicle (control). G_1 and G_2 fractions are identified; the arrowhead identifies the fraction corresponding to hyperdiploid cells.

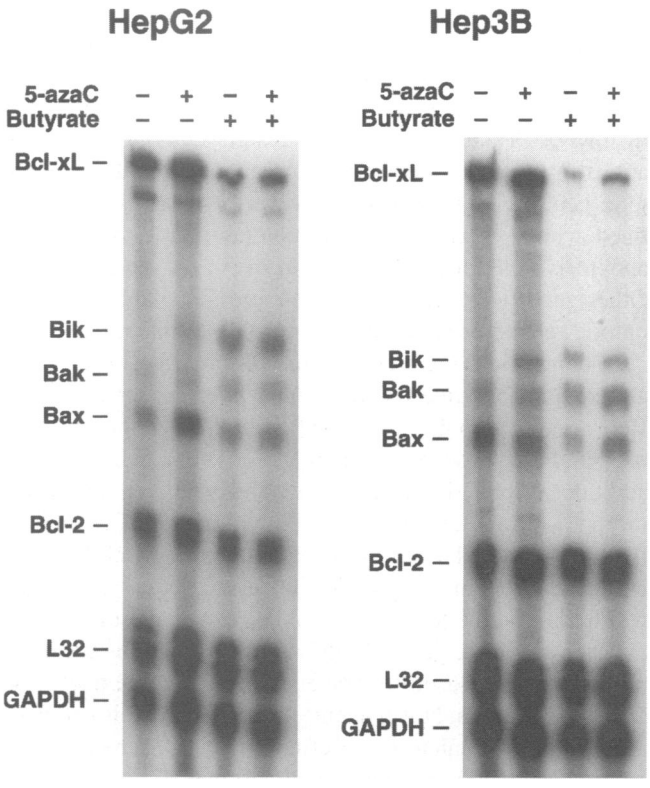

Figure 6. RNase protection analysis. RNA was extracted from HepG2 and Hep3B cells either untreated or treated with 5-azaC, butyrate, or 5-azaC and butyrate and analyzed by RNase protection using a multiprobe (hApo-2) that detects members of the Bcl-2 family. The constitutively expressed genes L32 and GAPDH are included to assess relative RNA loading equality.

genes, were unchanged with treatment, confirming the relative equality of loading.

Protein levels were next assessed by immunoprecipitation and Western blot (Fig. 7). Bax protein expression was not changed after treatment; Bcl-2 protein levels were decreased with treatment with both 5-azaC and butyrate, despite no change in mRNA levels. An attempt was made to analyze Bik protein levels, but we could not detect the protein despite several attempts.

Collectively, mRNA levels of several apoptotic genes were altered with various treatments, suggesting that cellular levels of these proapoptotic and antiapoptotic genes may be important for the morphologic and apoptotic changes noted after treatment with 5-azaC and butyrate. The fact that protein levels did not necessarily correlate with mRNA levels suggests different mechanisms of cellular regulation. Future studies are required to assess the apparent inconsistency between mRNA and protein levels noted and to define the role of these proteins, as well as other candidate genes, in the cellular changes noted in these cells after treatment.

DISCUSSION

We compared the effects of 5-azaC and butyrate on the human liver cancer cell lines HepG2 and Hep3B. The effects of these agents were variable. 5-azaC treatment resulted in cells that exhibited a more differentiated morphology, with arrest in the G_2 phase of the cell cycle. 5-azaC treatment also triggered apoptosis of both liver cancer cell lines, with a more pronounced effect noted in HepG2 cells. In contrast, a differentiated morphology was not apparent with butyrate treatment, but apoptosis occurred in both cell lines. The combination of these agents produced differential effects on the cell cycle distribution of both cell lines without notable increases in apoptosis compared with either agent alone.

The cytidine analog 5-azaC incorporates into the DNA, resulting in the hypomethylation of various genes and differentiation of certain cell lines. For example, 5-azaC stimulates expression of myoD, a member of a family of myogenic determination genes, and the differentiation of myogenic cell lines.[28] 5-azaC has also been used as a potent anticancer agent in a limited number of tumors, such as myeloid leukemias.[29,30] Murakami et al.[19] demonstrated that 5-azaC at low concentrations (2 to 6 μM) induced apoptosis only in HL-60 cells in the G_1 phase of the cell cycle; higher concentrations (8 to 40 μM) of 5-azaC resulted in apoptosis in cells without regard to cell cycle specificity. In another study, He and Rao[31] found that the cellular differentiation of HL-60 was increased after 4 days of 5-azaC treatment; conversely, DNA methylation levels were decreased.

Recent studies have focused on the induction of cell cycle arrest using agents that target specific genes.[32,33] This approach may prove to be beneficial in the treatment of certain cancers. Our results demonstrated that 5-azaC induced a G_2 phase arrest in both HepG2 and Hep3B cells. The effects of 5-azaC on the cell cycle may be variable, because treatment of NIH-3T3 fibroblasts with 5-azaC induced a reversible G_1 arrest; however, 5-azaC produced a persistent G_2 arrest in amniotic fluid-derived fibroblastlike cells in the same experiment.[34] These contrasting effects of 5-azaC may reflect intrinsic differences in methylation patterns. Further research is required to define the molecular mechanisms leading to the alterations in the cell cycle induced by 5-azaC.

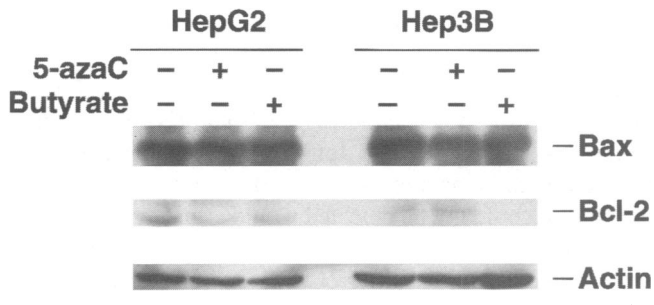

Figure 7. Western blot analysis. Protein was extracted from HepG2 and Hep3B cells after treatment with 5-azaC or butyrate; untreated cells served as control. Protein was immunoprecipitated with antibody to Bax or Bcl-2 and analyzed by gel electrophoresis and visualized using ECL detection. Protein samples were also probed with antibody to actin to ensure intact protein samples.

Our findings demonstrate that 5-azaC induces differentiation, G_2 cell cycle arrest, and apoptosis in human liver cancer cells and suggest that agents like 5-azaC may be useful adjuncts in the treatment of primary liver cancer.

Treatment of both liver cancer cell lines with butyrate induced apoptosis but did not produce demonstrable changes in cellular morphology that would be consistent with a more differentiated-appearing phenotype. Conversely, butyrate has been shown to produce changes in several cancer cell lines that are consistent with a reversion to a more differentiated morphology. For example, butyrate induces differentiation in the human K562 erythroleukemia cell line by apparently activating MAP kinase and in the human colon cancer cell lines Caco-2, HT-29, COLO205, and SW620 by decreasing cytoskeleton-associated tyrosine kinase activity.[20,35,36] In addition, butyrate treatment results in the differentiation of human sarcoma and ovarian cancer cell lines, as well as the mouse fibroblast cell line NIH-3T3.[21,37,38]

Butyrate has also been shown to trigger apoptosis in various human cancer cell lines such as colon, breast, and lung cancers, as well as retinoblastoma and leukemic cells.[39–46] Our results confirm that butyrate can also induce apoptosis in the human liver cancer cell lines HepG2 and Hep3B. Although butyrate has been shown to produce cell cycle arrest of human colon cancer lines and rat hepatoma cells,[47,48] we could not demonstrate a significant effect of butyrate on the cell cycle distribution of either HepG2 or Hep3B.

To begin to assess the molecular mechanisms underlying the changes in morphology and proliferation in response to 5-azaC and butyrate, RNase protection assays (to evaluate changes in mRNA levels) and Western blots (to evaluate proteins levels) were performed. We evaluated members of the Bcl-2 family that either suppress (*e.g.*, Bcl-2, Bcl-xL) or promote (*e.g.*, Bik, Bax, Bak) apoptosis. The proapoptotic protein Bik shares only the BH3 domain with other Bcl-2 family proteins.[49] In contrast, Bax contains three conserved domains, BH1, BH2, and BH3, which are similar to Bcl-2, as well as the characteristic C-terminal transmembrane proteins.[10] Bik promotes apoptosis by complexing with various antiapoptotic proteins such as Bcl-2 and Bcl-xL. Bik can also sensitize cells to the induction of apoptosis by the Fas antigen.[50,51]

Treatment of both HepG2 and Hep3B cells with 5-azaC or butyrate resulted in an increase of Bik mRNA expression, suggesting a possible role for this gene in the apoptosis induced by these agents. However, the fact that Bik protein expression could not be detected would argue against Bik as an important factor in this process. Expression of the Bcl-2 protein was decreased after 5-azaC or butyrate treatment, but there was no apparent change in mRNA levels, suggesting regulation at the translational or posttranslational level. A previous study noted that butyrate-induced apoptosis in a human breast cancer line was closely linked to a decrease in Bcl-2 protein expression;[45] therefore, the decrease of Bcl-2 noted in our study may contribute to the apoptosis demonstrated by treatment with these agents. The expression of Bcl-xL mRNA was decreased after treatment, whereas the expression of Bax protein did not change despite decreases in mRNA. Taken together, our results demonstrate alterations in the expression of both proapoptotic and antiapoptotic genes after treatment. However, it remains to be defined which, if any, of these proteins play a role in the apoptosis noted after the addition of 5-azaC and butyrate. Other genes (*e.g.*, cell cycle-related proteins and other apoptotic-related proteins) may contribute to this process.

In conclusion, our study evaluated the effects of 5-azaC and sodium butyrate on the human liver cancer cell lines HepG2 and Hep3B. Treatment with 5-azaC or butyrate triggered the process of apoptosis in both cell lines. In addition, 5-azaC induced a more differentiated-appearing morphology and arrest of both HepG2 and Hep3B cells in the G_2 phase of the cell cycle. Proteins of the Bcl-2 family may play a role in this process; however, further studies are required to define the specific role of these proteins as well as other candidate genes. Products of the apoptotic pathway may prove to be useful therapeutic targets in the treatment of hepatic cancers. Novel agents that can target this pathway are required to enhance the effect of current chemotherapeutic agents and prolong patient survival.

Acknowledgments

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Discussion

DR. R. SCOTT JONES (Charlottesville, Virginia): Thank you, Dr. Wells, Dr. Copeland, Ladies, and Gentlemen.

I'd like to compliment Dr. Evers and his associates on this fine piece of experimental laboratory work. Basically, they have demonstrated that the two agents investigated altered cell growth and proliferation in liver cancer cells in culture and went on to analyze and to provide additional information so that we could understand how the agents worked.

I have two questions. One is to ask Dr. Evers if he would give us perhaps a little bit better or more complete description of the cells that he used in this particular study. As you know, most liver