COLON CANCER IS ONE OF the most common cancers in western society and the second leading cause of cancer-related mortality in the United States (36). Despite some data to the contrary (37), numerous epidemiological and animal studies suggested a pivotal role of dietary fiber in reducing the incidence of colon carcinomas (9, 23, 41), and it appears that at least part of this protective effect relates to the microbial fermentation of fiber into short-chain fatty acids. For example, the four-carbon short-chain fatty acid butyrate is a metabolic by-product of dietary fiber and was shown to possess antiproliferative and prodifferentiating properties in cell cultures (24, 34) and to inhibit the growth of colon cancer in animal models (30, 43). In addition, butyrate was documented to induce apoptosis in several colon cancer cell lines (17, 18).

Although its precise mechanisms of action are poorly understood, butyrate was shown to exert several modulatory effects on nuclear proteins, including selective inhibition of histone phosphorylation, hypermethylation of cytosine residues in DNA, and histone hyperacetylation. Of these various butyrate effects, hyperacetylation of core histones is the best characterized and appears to be an important mechanism by which gene transcription is regulated (12, 19, 38). Similar to the effects of butyrate on nuclear histones, trichostatin A (TSA), a fungistatic antibiotic purified from Streptomyces platensis, induces a marked accumulation of highly acetylated histones by strongly inhibiting the activity of histone deacetylase (49). At low concentrations, TSA was shown to induce differentiation in Friend leukemia cells and cell cycle arrest in normal rat fibroblasts (48, 50). Therefore, TSA appears to be useful in analysis of the role of histone hyperacetylation in regard to butyrate-mediated cellular proliferation and differentiation.

Previous studies from our laboratory showed two distinct mechanisms by which butyrate mediates growth inhibition in colon cancer cells, one involving histone hyperacetylation and induction of the cell cycle inhibitor p21 (3) and the other relating to impairment in growth factor responsiveness (4). In the present work, we have further examined the role of histone hyperacetylation in regard to cellular growth, differentiation, and apoptosis in colon cancer cells.

MATERIALS AND METHODS

Cell culture and experimental design. HT-29 human colon carcinoma cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were main-
Effects of Histone Hyperacetylation on Colon Cancer Cells

G483

tained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and penicillin-streptomycin (100 U/ml) in 95% O_2-5% CO_2 at 37°C. Treatments of HT-29 cells with hyperacetylating agents were as follows: 5 mM sodium butyrate (NB), 0.3 mM TSA (T), or saline control for 24 h. Alternatively, three doses of 0.3 mM TSA (TR treatment) were added sequentially to HT-29 cells every 8 h for 24 h. HCT116 p21 wild-type (+/+ ) and p21-deleted (−/−) cells were kindly provided by B. Vogelstein (44). These cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin (100 U/ml) in 95% O_2-5% CO_2 at 37°C. They were treated with 1 mM NB or 0.15 mM TSA for the times indicated. Higher concentrations of both acetylating agents as indicated for 24 h, nuclear histones were prepared by acid extraction with 0.2 M H_2SO_4, recovered by acetone precipitation as described previously (49), and separated by slab gel electrophoresis using a 10-cm acid-urea-Triton gel (5% acetic acid, 8 M urea, 0.4% Triton X-100, 0.1% N,N'-methylene-bis-acrylamide, 15% acrylamide) with incorporation of a 3-cm upper gel (5% acetic acid, 10 M urea, 5% acrylamide/bis-acrylamide, 30% acrylamide). Fifty micrograms of histones were incubated with the same volume of loading buffer (10 M urea, 5 M NH_4OH, 10 mM dithiothreitol) for 5 min, and a one-eighth volume of 1% pyronine G (Sigma) in glacial acetic acid was added. The mixture was loaded onto the upper stacking gel and electrophoresed overnight in 0.2 M glycine and 1 M acetic acid. Gels were stained with Coomassie brilliant blue R-250 (Sigma), destained in 40% methanol and 10% glacial acetic acid, dried, and photographed.

RESULTS

NB and TSA induce histone H4 hyperacetylation. Both NB and TSA were shown to inhibit histone deacetylation, leading to hyperacetylation of selective histone proteins such as histone H4 (7, 49). Figure 1A illustrates the time course of H4 hyperacetylation induced by NB and TSA in HT-29 cells. The Coomassie blue-stained protein gel shows that the unacetylated and monoacetylated H4 proteins predominate in the untreated cells, but increased acetylation of H4 histones was observed by 4 h of NB and TSA treatments. There was a decrease in unacetylated and monoacetylated H4 proteins with concurrent increases in di-, tri-, and tetra-acetylated forms. By 24 h, H4 hyperacetylation persisted in the NB group but returned to baseline in the T group. In contrast, repetitive doses of TSA (TR) induced a state of prolonged H4 hyperacetylation similar to that seen with NB treatment. This state of hyperacetylation in the TR group was maintained over the entire 24-h period as depicted in Fig. 1B.

Effects of histone hyperacetylating agents on growth of HT-29 cells. Figure 2A shows the growth inhibitory effects of NB and TSA on HT-29 cells. At 24 h, NB, T, and TR treatments produced a growth suppression of 27 ± 0.4%, 21 ± 0.4%, and 24 ± 0.3%, respectively, compared with control cells. Figure 2B shows the time course for these effects.

Induction of p21 mRNA by NB and TSA. Butyrate and TSA were previously shown to rapidly induce p21 mRNA levels in HT-29 cells (3). Figure 3A depicts a detailed time course experiment demonstrating that the p21 mRNA is induced by TSA as early as 1 h, peaking at 6 h but returning to baseline levels by 24 h. In contrast, Fig. 3B demonstrates the persistent induction of p21 mRNA expression (at 24 h) with repetitive TSA or NB administration, whereas p21 levels are at baseline by 24 h after a single dose of TSA (T).
Effects of transient vs. prolonged histone hyperacetylation on HT-29 cell differentiation. Treatment of HT-29 cells with butyrate was shown to induce a differentiated phenotype characterized by IAP induction and NKCC1 downregulation (25, 42). We therefore tested the effects of TSA on the expression of NKCC1 and IAP mRNAs in HT-29 cells. As shown in Fig. 5, both NB and repetitive TSA treatment induced IAP mRNA expression by 24 h, whereas IAP mRNA was undetectable in control or T cells. On the other hand, NKCC1 mRNA was expressed at high levels under basal conditions and downregulated in response to 24-h treatment with NB or repetitive TSA doses. Similar to what was seen in the case of IAP, the decrease in NKCC1 expression was not observed in cells treated with a single dose of TSA, i.e., the NKCC1 mRNA remained at high basal levels.

Prolonged histone hyperacetylation is required for induction of apoptosis in HT-29 cells. As shown in Fig. 6, NB and TR treatments produced significant increases in overall numbers of apoptotic cells at both 24 and 48 h (25- and 10-fold, respectively; \( P < 0.001 \)) as determined by propidium iodine staining. The percentage of apoptotic cells induced by a single dose of TSA at 24 h was not significantly different from that observed under basal conditions. As expected, there was a significant increase in the number of apoptotic cells in the floating population of cells treated with NB for an extended period of time (48 h), whereas a more significant number was noted in the adherent population at an earlier time point (24 h). Similar results were seen for the TR-treated cells, although more apoptotic cells remained adherent and the overall number of apoptotic cells in the floating population at 48 h was lower. These findings were confirmed by Hoechst staining (data not shown).

Electron microscopy was then used to confirm the morphological features of apoptosis with repetitive TSA treatment for 24 h. As shown in Fig. 7A, control HT-29 cells form a distinctive monolayer that lacks intercellular spaces or brush borders. The nuclei of these untreated cells show randomly dispersed euchromatin. In contrast, Fig. 7B (TR treatment) reveals several key features of apoptosis including reduction of cellular volume, condensation of chromatin, margination of euchromatin, and blebbing of plasma membrane. Similar results were seen with NB treatment (not shown).

We (3) showed previously that the growth arrest induced by either NB or TSA requires the cell cycle inhibitor p21. We now sought to determine whether p21 was important in the apoptosis induced by prolonged histone hyperacetylation. For these studies, we used HCT116 colon cancer cells in which the p21 gene had been deleted (\(-/-\)). Figure 8 shows that butyrate induced similar levels of apoptosis (5-fold over control, untreated; \( P < 0.005 \)) in both +/+ and \(-/-\) cell lines. This finding suggests that p21 is not required for the apoptosis induced by prolonged histone hyperacetylation.

**DISCUSSION**

Previous studies suggested that a diet high in fat and protein may promote colon cancer, whereas increased fiber and complex carbohydrates in the diet may protect against colon cancer (9, 23, 25, 41, 42). The production of butyrate, a metabolic by-product of bacterial fermentation of fiber in the colon, appears to be at least
Fig. 2. Effects of butyrate and TSA on HT-29 cell growth. Cells were incubated for 24 h in culture medium with 5 mM sodium butyrate (NB), 0.3 μM TSA (T), or repetitive TSA doses (TR), and FACS analysis was performed. A: relative S phase fraction at 24 h for all groups was determined by flow cytometric measurement of DNA content. Cell number is shown on the y-axis and DNA content on the x-axis. B: time course for growth inhibition by NB and TSA (n = 4, P < 0.001).
partly responsible for the protective effects of dietary fiber in terms of colon carcinogenesis. In vivo studies have indicated that increased colonic butyrate levels correlate with a decreased incidence of colon cancer (6, 30, 31), and in vitro studies have shown that butyrate inhibits cell proliferation, induces differentiation, and promotes apoptosis (17, 18, 46). In addition, recent studies from our laboratory (4) also showed that butyrate inhibits EGF responsiveness in colon cancer cells.

We chose to use HT-29 cells, a human colon cancer-derived cell line, to study the role of histone hyperacetylation in the regulatory processes of cellular proliferation, differentiation, and apoptosis. Since the early discovery of histone acetylation by Allfrey and colleagues (2), this posttranslational modification has been correlated with the processes of chromatin assembly and gene transcription. Acetylation of core histones occurs at specific lysine residues in the flexible NH2-terminal tails, thereby disrupting DNA-histone interactions and allowing transcriptional activators and co-activators to access the DNA (40). The steady state of histone acetylation is controlled by the equilibrium of two distinct families of enzymes, histone acetyltransferases and histone deacetylases.

Yoshida et al. (49) described a noncompetitive histone deacetylase inhibitor, (R)-trichostatin A (TSA), as a potent, stereospecific hyperacetylating agent. Like butyrate, TSA was reported to cause growth inhibition and differentiation in some cell lines (48, 50). It is likely that the common effects of butyrate and TSA occur as a consequence of histone hyperacetylation, although other mechanisms of action may be operative. Importantly, elevated levels of butyrate in rats fed a high-fiber diet were correlated with histone hyperacetylation in colonocytes, suggesting that this phenomenon also occurs in vivo (6).

The processes of growth arrest and terminal differentiation are associated with increased levels of the cell cycle inhibitor p21 (14, 39). Expression of the p21 protein in vivo was detected by immunohistochemistry in upper crypt and lower villus cells, an area associated with...
with enterocyte differentiation (16). Activation of p21 was reported to occur before terminal differentiation and apoptosis in human monocytic cells (47), further suggesting a link between p21 induction and differentiation and apoptosis. We (3) previously documented that butyrate-induced growth inhibition of human colon cancer cells was mediated by p21 and that histone hyperacetylation is at least partly responsible for its induction. This model is further supported by the present findings, which suggest that transient p21 induction with a single dose of TSA is capable of inducing growth arrest.

Previous studies showed that butyrate decreases the sensitivity of cells to growth stimuli by blunting various components of the growth factor signaling pathway. For instance, expression of both the ras and c-myc proteins has been shown to be downregulated by butyrate in HT-29 cells (5, 13). Furthermore, we (4) showed that the induction of c-fos and c-jun protooncogenes in response to EGF was minimal in butyrate-treated cells. Our present results advance these initial observations, demonstrating that the growth-stimulatory effects of both EGF and IGF-I are abolished in the setting of prolonged histone hyperacetylation. The relatively small increase in percent S phase by EGF and IGF-I in control cells is likely related to the presence of growth factors already present within the serum-supplemented media under control conditions. Indeed, we have seen a more dramatic proliferative response to EGF and IGF-I in serum-starved cells. However, the finding that NB and TR treatments completely blocked these increases, whereas a single dose of TSA did not (and actually increased the S phase), is particularly significant. Interestingly, the lack of growth factor responsiveness was also seen in the case of replenishment of culture medium with serum (data not shown), suggesting that the effects of butyrate in regard to growth factor responsiveness are not limited to tyrosine phosphorylation pathways but rather occur at a fundamental level encompassing a variety of growth stimuli. Inhibition of the growth stimulus may be related to downregulation of growth factor receptors (4), but other components of the growth factor-associated signaling pathways may also be affected. In contrast, an increased sensitivity to growth factors in the context of transient histone hyperacetylation may be caused by a synchronized G1 progression following recruitment of cells in the G0 phase. Clearly, histone hyperacetylation appears to have a significant modulatory role in terms of growth factor responsiveness. The precise molecular mechanism(s) by which histone hyperacetylation blocks growth factor responsiveness will require further investigation.

The role of growth factors in the induction of cellular transformation has been implicated in colon carci-
EFFECTS OF HISTONE HYPERACETYLATION ON COLON CANCER CELLS

Constitutive activation of mitogenic signaling by growth factor receptors as oncogene products is intimately associated with the generation of malignant cells, and autologous production of a given growth factor could result in a growth advantage for the cell. These effects require the maintenance of cellular responsiveness to growth factors. Therefore, the fact that both butyrate and TSA are capable of inducing growth factor unresponsiveness suggests that strategies targeting tumor cells with hyperacetylation agents could provide a means for inhibiting colon carcinogenesis.

The basolateral Na-K-2Cl cotransporter NKCC1 is an integral component of the intestinal crypt secretory apparatus involved in the regulation of transepithelial Cl⁻ secretion. NKCC1 mRNA is confined to the crypt cells in vivo, and its expression has been shown to be downregulated by butyrate treatment in vitro (28). In contrast, the expression of the brush border enzyme IAP is confined to villus enterocytes in vivo and is dramatically induced in butyrate-treated HT-29 cells (22). Thus butyrate treatment of HT-29 cells induces a pattern of differentiation resembling that seen along the crypt-villus axis of the intact mammalian gut. The basal growth inhibition and the lack of growth factor responsiveness seen in butyrate-treated HT-29 cells are also features consistent with the differentiation phenotype.

We (22, 28) showed previously in HT-29 cells that both induction of IAP and downregulation of NKCC1 by butyrate are blocked by protein synthesis inhibitors. These findings suggest a model in which butyrate induces the expression of one or more proteins responsible for the differentiation-specific changes in gene expression. Our present data, which indicate the requirement for prolonged histone hyperacetylation in colon cancer cell differentiation, are in keeping with this model and are consistent with the notion that differentiation is likely a multistep process.

In the human colon, apoptosis was shown to occur at the superficial crypt compartment, and its regulation appears to play an important role in tissue homeostasis as well as the pathogenesis of colon cancer (33). The protective role of butyrate in colon cancer was attributed, in part, to its ability to induce apoptosis (18, 20, 27, 29). Although the precise mechanisms by which apoptosis is triggered in cells remain elusive, it is believed that healthy cells constitutively express the molecules required for their own destruction and that the function of cell death genes is to activate these molecules (45). Conceivably, cell death genes are normally silenced in hypoacetylated, transcriptionally inactive domains, where histone deacetylase activity is high, and these areas may be rendered more accessible to transcription factors by the suppression of deacetylation (35). We were interested in assessing the degree of apoptosis in HT-29 cells in relation to the extent of histone hyperacetylation. Our findings indicate that apoptosis was apparent as early as 24 h after NB or TR treatment, suggesting an intimate link between prolonged histone hyperacetylation and the induction of apoptosis. Hague et al. (18) reported that a significant increase in the proportion of floating cells after 4 days of butyrate treatment was caused by the induction of apoptosis. In that study, minimal apoptosis was seen in the adherent cells (18). In our studies, a significant increase in apoptosis was seen in the adherent groups of both NB- and TSA-treated cells as early as 24 h. Our results may reflect a greater sensitivity of FACS analysis in determining apoptosis. Previous studies documented that both butyrate and TSA induce apoptotic cell death by a process that is dependent on the inhibition of histone deacetylase and new protein synthesis (26, 32). Medina et al. (32) showed that apoptosis in LIM 1215 colon cancer and Jurkat lymphoid cancer cells occurs with a single dose of butyrate and depends on the production of the catalytically active effector protease of the caspase-3 cell death gene. In the present studies, we show that prolonged histone hyperacetylation by a single dose of butyrate or repeated doses of TSA is required for the apoptotic effect in HT-29 cells and demonstrate the morphological characteristics of apoptosis induced by TSA. Despite a transient increase in histone hyperacetylation at 6 h, a single dose of TSA was not sufficient for induction of apoptosis, even when we examined cells at the 6-h time point (data not shown). The dose of TSA used in our studies is comparable to that used by others for induction of apoptosis (32). It is therefore likely that prolonged histone hyperacetylation facilitates apoptosis by increasing the expression of one or more cell death genes.

Although the precise role for p21 in the various processes described in the present studies has not been extensively studied, it appears that, in contrast to the growth arrest, which is p21 dependent (3), p21 may not be required for apoptosis because butyrate induced apoptosis to the same extent in both HCT116 wild-type and p21-deleted colon carcinoma cells. Whether p21 plays a role in differentiation and/or growth factor unresponsiveness will require further study.

In conclusion, the present study highlights the critical role of histone hyperacetylation in regulation of colon cancer cell growth, growth factor responsiveness, differentiation, and apoptosis. It is hoped that future studies will lead to the identification of those specific gene products that are regulated by histone hyperacetylation and function in these diverse, but linked, cellular processes.

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REFERENCES


