SCFAs such as butyrate are produced in the colon by fermentation of dietary fiber (9, 10) and have well known effects on the stimulation of differentiation of colonic carcinoma cells in culture (11–22). However, the data of Hague et al. are based on the morphological evaluation of cells which detach from the culture surface, and there was no assay of the status of differentiation of the apoptotic cells (8). We have investigated apoptosis and cell shedding in cultured colonic carcinoma cell lines and also found that the bulk of cells shed into the media exhibit properties of apoptosis. Furthermore, using ALP activity as an index of differentiation (12, 14, 16, 18–22), a sensitive DNA-labeling technique (23), and careful quantitation of the degradation of high molecular weight DNA to lower molecular weight fragments which characterize apoptosis (24), we demonstrate that both the spontaneously shed apoptotic and SCFA-induced cells are linked to a more differentiated phenotype and that the process of apoptosis is likely initiated in the adherent cell population. Finally, in addition to studying the effects of C4, we have also investigated the effects of two derivatives, isoC4 and fluoroC4, and find them to be ineffective inducers of either differentiation or apoptosis. This is similar to the results we obtained on induction of mitochondral gene expression and mitochondrial activity by the SCFAs (25) and suggests that the effects of SCFAs are linked to their structure and most likely their utilization. Thus, as we have previously suggested (25–28) and as supported by the data herein and that of Hague et al. (8), SCFAs generated by dietary fiber consumption may be important modulators of the biochemical and cellular events which contribute to the relative risk for development of colon tumors.

MATERIALS AND METHODS

Cell Culture

The HT29 and SW620 colon cancer cell lines were obtained from the American Type Culture Collection and maintained as described previously (25).

For studies of apoptosis and differentiation of cells under standard, uninduced conditions, postconfluent cultures were provided with fresh media and shed cells were harvested from the conditioned media following 6, 16, 24, 48, and 72 h of incubation. Adherent cells were obtained from the same flasks, at the same time points, by scraping.

For SCFA induction, postconfluent cultures were fed 24 h before treating with SCFAs for 72 h. Stock sodium butyrate or free isobutyric or heptfluorobutyric acids (1 mM; Sigma Chemical Co.) were added to tissue culture media immediately before use to produce final concentrations of 5 mM.

Again, cells which accumulated in the conditioned media were harvested by centrifugation and adherent cells were obtained from the same flask by scraping.

Apoptosis

Apoptosis was evaluated in both shed and adherent cell populations by analyzing DNA fragmentation. Three methods were used.

Quantitation of DNA Fragmentation. The percentage of fragmented DNA in shed and adherent cells was determined by comparing the amount of DNA in a low molecular weight fraction to the total DNA using the method of Sellins and Cohen (24).

Visualization of DNA Fragmentation. A portion of the low molecular weight DNA fraction was precipitated overnight with 0.5 M NaCl and 50% isopropyl alcohol (24). Approximately 1 μg of DNA was electrophoretically...
size fractionated through 1.7% agarose gels containing 3.5 μg/ml ethidium bromide. *Hae*III-restricted φX174 DNA served as a size reference.

[^32]PdCTP End Labeling of Apoptotic DNA. The DNA fragmentation characteristic of apoptotic cell death was confirmed by[^32]PdCTP incorporation as described by Rosl (23). Approximately 1500 cpm of labeled DNA were size fractionated through 1.7% agarose gels, dried on Whatman No. 3MM paper, and exposed to X-ray film. *Hae*III-restricted φX174 DNA was included as a size marker.

**Index of Number of Cells Shed**

Cells which were shed into the conditioned media following SCFA treatment or uninduced culture conditions were harvested by centrifugation. DNA was extracted and quantified as described above (24). Total DNA/ml of conditioned media was determined as an index of cell shedding.

**Alkaline Phosphatase Activity**

The specific activity of ALP was determined as an index of differentiation of colonic carcinoma cells to a more normal phenotype. Cells were lysed with 0.25% sodium deoxycholate (19) and total protein was determined (29). Specific ALP activity was quantified using Sigma procedure 245 (30).

**Statistical Analyses**

At least three independent determinations of ALP activity, percentage of fragmentation, and cell shedding in uninduced and SCFA-induced cells were compared with two-sample t tests using individual groups and pooled variances (GB-Stat Computer-Aided Statistics, Ver. 1.0; Dynamic Microsystems).

**RESULTS**

**Colonic Carcinoma Cells Which Accumulate in the Conditioned Media during Standard, Uninduced Culture Conditions Are Apoptotic.** Hague et al. (8) have documented that the majority of cells that are shed into the media of colonic tumor cells grown in culture are morphologically apoptotic and exhibit ladders of DNA cleaved to nucleosomal size fragments which characterize this process (31, 32). We have confirmed and extended this observation.

Postconfluent cultures of the human colonic carcinoma cell lines HT29 and SW620 were provided with fresh media and then incubated for 72 h. Cells which accumulated in the conditioned media were harvested by centrifugation and adherent cells were obtained by scraping the flasks. Low molecular weight DNA fractions were isolated and size fractionated. As shown in Fig. 1 (control lanes, *A* panels), bands in multiples of 212 ± 28 (SD) or 180 ± 10 base pairs were seen in this DNA when visualized by ethidium bromide staining or[^32]P end labeling of the fragmented DNA, respectively.

Visualization of fragmentation in the adherent population by ethidium bromide staining was difficult because, as discussed below and shown elsewhere (8), only a small amount of the total DNA in the adherent cells is a low molecular weight. However, by specifically end labeling DNA fragments generated by apoptosis (23), it is clear that fragmented DNA is also present in adherent cells following 72 h of incubation under standard, uninduced conditions and appears as oligonucleosomal size fragments characteristic of apoptotic cells (Fig. 1, *B* panels).

We next asked whether apoptotic DNA fragmentation in shed cells occurred coincidentally with extrusion or during the 72-h period of detachment. Again, postconfluent cultures of HT29 and SW620 cells were fed, and cells that accumulated in the conditioned media following 6, 16, 24, 48, and 72 h of incubation under standard, uninduced conditions were harvested. Adherent cells were also obtained at the same time points by scraping the flasks. Low and high molecular weight fractions of DNA were isolated and the percentage of DNA recovered as low molecular weight was determined. As shown in Fig. 2, although the percentage of fragmented DNA present in the shed cells was significantly greater than in the adherent cells at
each time point (P < 0.05), fragmentation did not significantly change within either the shed or the adherent cell populations during the 72-h time course. Thus, in the HT29 and SW620 cell lines, maximal DNA fragmentation of approximately 65% is seen in shed cells and a consistent low level of fragmentation (about 3.0%) is seen in adherent cells. The value for the percentage of DNA fragmentation in shed cells agrees with the morphological data which showed that the percentage of apoptotic cells in the shed population ranges from 67 to 88% for different colonic carcinoma cell lines (8).

The consistent low level of apoptotic DNA fragmentation in the adherent populations combined with the shedding of apoptotic cells, similar to the extrusion of apoptotic cells into the colonic lumen (2, 3), predicted that the total number of cells accumulating in the conditioned media would increase over time, as described previously (8). However, because of the alterations in cellular morphology that accompany apoptosis (33, 34), both manual and automated counting of the shed cells were found to be unreliable. Therefore, we determined the total amount of DNA (high plus low molecular weight) in conditioned media as an index of the number of cells shed. As shown in Fig. 3, as expected, the number of cells which accumulated in the conditioned media increased in each cell line following 6–72 h of incubation.

Uninduced Apoptotic Colonic Carcinoma Cells Exhibit a More Differentiated Phenotype than Adherent Cells. Since apoptotic cells are seen among terminally differentiated phenotypes and are shed in vivo, we next asked if apoptotic shed cells generated during uninduced culture conditions exhibited a more differentiated phenotype than adherent cells, the majority of which have not initiated apoptosis. As described under "Discussion," the evaluation of differentiation of colonic epithelial cells is complex. We used a well established marker of a more differentiated phenotype, increased specific activity of ALP (12, 14, 16, 18–22). Cells were harvested from conditioned media following 6–72 h of incubation; adherent cells were obtained at the same time points from the same flasks. In both HT29 and SW620 cells, at each time point, the ALP activity in the apoptotic shed population is substantially higher than that in the adherent population (Fig. 4; P < 0.05).

Therefore, at least some cells in the HT29 and SW620 human colonic carcinoma lines undergo spontaneous, uninduced differentiation and apoptosis and accumulate in the conditioned media.

Butyrate, but not Its Derivatives, Potentiates Differentiation and Apoptosis in Human Colonic Carcinoma Cells. Because of the potential physiological relevance of SCFAs as natural effectors of colonic cell differentiation (35–41), we have previously investigated the specificity of SCFA induction of mitochondrial gene expression and mitochondrial activity, components of induction of colonic cell
to stimulate apoptosis in the HT29 and SW620 cells. This indeed was the case. Consistent with the previously published work, 72 h after continuous treatment with butyrate, the percentage of fragmented DNA in the adherent cells increased 2–4-fold in the two cell lines and was statistically significant for each line (P < 0.05; Fig. 6). The biological relevance of such increases in a small population of apoptotic cells is addressed under “Discussion.” This fragmented DNA exhibited the ladder characteristic of apoptotic cells when the low molecular weight DNA was specifically end labeled, fractionated by electrophoresis, and visualized by autoradiography (Fig. 1, B panels).

Fig. 6 also shows that the percentage fragmentation does not increase in the shed cells, a finding that corroborates previous work demonstrating that the percentage of shed cells exhibiting an apoptotic morphology is not altered by butyrate treatment (8). The shed cell population does not respond in terms of DNA fragmentation since this population represents the accumulation of end-stage cells which have initiated the process earlier.

These results demonstrate that butyrate specifically affects the adherent cell population, eliciting an increase in the percentage of cells progressing to a more differentiated phenotype (Fig. 5), initiating apoptosis (Fig. 6), and subsequently being shed into the media. Indeed, following butyrate treatment we found significant (P < 0.05) increases in the amount of DNA in the media (an index of the number of cells shed) of 1.5- and 3.3-fold in HT29 and SW620 cultures, respectively (data not shown), as has been reported (8).

differentiation (25, 26). Here, we extended these studies to include enhanced ALP activity and potentiation of apoptosis in HT29 and SW620 cells by butyrate and two of its derivatives. Unbranched C4 is generated during fermentation of fiber and is found at concentrations as high as 15 mM in the colonic lumen (42). Branched isoC4, produced during the metabolism of valine, is present in the colonic lumen in small quantities (43). Finally, fluoroC4 is a synthetic analogue of butyrate which is not readily metabolized due to the high fluorine-carbon bond energy (44, 45).

Postconfluent cell cultures were treated with these SCFAs at 5 mM for 72 h. As shown in Fig. 5, C4 significantly increased ALP in both HT29 and SW620 cells. The effect was especially dramatic in HT29 cells (note change of scale, Fig. 5, top), consistent with reported differences in butyrate-induced activity among these cell lines (12, 16, 19). Although isoC4 also significantly increased ALP activity in HT29 cells, levels were approximately 65-fold less than those mediated by C4, and isoC4 did not alter ALP activity levels in SW620 cells. Furthermore, fluoroC4, the nonmetabolizable analogue of butyrate, had no effect on the activity of ALP in either of the cell lines.

It has been reported that butyrate can stimulate the extent of apoptosis in colonic tumor cell lines (8). If apoptosis is linked to a more differentiated phenotype then we would expect butyrate, but not the two derivatives which are relatively ineffective in inducing ALP,
cells normally undergo a sequence of events which includes proliferation, differentiation, apoptosis, and extrusion. In these studies we have shown that these apoptotic cells exhibit increased ALP activity, an index of a more differentiated phenotype. Therefore, at least some of the cells in each of these two cell lines retain active pathways of differentiation and apoptosis and accumulate in the conditioned media, recapitulating in vivo processes. Since these cell lines are not clonal, it is not yet known whether these apparently spontaneously initiated pathways represent stochastic events or define inherent potentials of specific populations of transformed colonic epithelial cells (6).

The relationship between differentiation and apoptosis in colonic carcinoma cell lines is likely to be complex, as illustrated by the data in Figs. 5 and 6. Although butyrate is a much better inducer of ALP activity in HT29 cells than in SW620 cells, DNA fragmentation is induced in both to approximately the same extent. However, it is important to note that ALP is used as only a single marker of a more differentiated phenotype, and it would not be correct to interpret the data as indicating that the induced HT29 cells were “better” differentiated than the SW620. For example, mucinous tumor cell lines produce vast amounts of colonic cell mucin and express the MUC2 gene to a far higher extent than do more normal, nontumorigenic lines differentiated along the goblet cell lineage (46, 47). However, these mucinous cell lines certainly cannot be considered better differentiated than normal goblet cells. Given the extensive heterogeneity in genetic alterations in colonic tumors (48–50), it is not surprising that colonic carcinoma cells exhibit widely differing responses to an inducer of differentiation. Of importance is the consistency in the data for both spontaneous and induced apoptosis for both cell lines. The population undergoing more extensive apoptosis expresses at least one differentiation marker (ALP) at a higher level: shed cells which are extensively apoptotic exhibit greater ALP activity than adherent cells (Fig. 4); butyrate-induced adherent cells show greater fragmentation and ALP than uninduced adherent cells (Figs. 5 and 6). Finally, consistent with this and demonstrating the specificity of these responses, derivatives of butyrate are ineffective in inducing either ALP activity or potentiation of DNA fragmentation.

A key question is then which aspects of differentiation and apoptosis are tightly linked and which genes control these processes. It has been demonstrated that expression of a wild-type p53 gene in a colonic carcinoma cell line can stimulate extensive apoptosis (51), but it has also been documented that both spontaneous and butyrate-induced apoptosis are independent of p53 pathways (8). Other candidates may include the c-myc gene, which is both overexpressed (52) and amplified at a low level in colonic tumors (53), and which can also affect apoptosis in other systems (54, 55), and the DCC gene, which may be related to cell adhesion (56, 57) and hence the shedding associated with apoptosis. There are likely a large number of genetic and environmental signals the integration of which determines the final balance of differentiation and apoptosis in the colonic mucosa, all of which can affect the process in culture to some extent.

It is not clear how the three-dimensional structure of the colonic crypt influences the access of cells to contents of the colonic lumen such as SCFAs, macro- and micronutrients, and growth factors. However, the stimulation of both differentiation and apoptosis by the SCFA butyrate is of interest. Organic acids generated as products of fiber fermentation by endogenous intestinal bacteria make up approximately 75% of the aqueous phase of the colonic contents (42). One-half of these acids have been structurally identified with butyric acid, accounting for >20% of the total by weight (42, 43). Small quantities of branched SCFAs are also found, including 4-carbon atom isobutyric acid, which is produced during the metabolism of the branched amino acid valine (42, 43). Normal colonic epithelial cells derive 60–70% of their energy supply from SCFAs (10). Therefore, depletion of colonic fatty acids, for example, following diversion of the fecal stream associated with colostomy (35, 36), is

Most important, Fig. 6 illustrates that unlike the increases seen with butyrate, there is no significant increase in the percentage of fragmented DNA in the adherent cells with isoC4 or the nonmetabolizable fluoroC4, consistent with their relative lack of effect on the differentiation marker ALP (Fig. 5). Further, neither isoC4 nor fluoroC4 increased the number of cells shed (data not shown). Thus, SCFA-induced increased activity of the differentiation marker ALP and apoptosis are associated with fatty acid structure and, most likely, utilization (see “Discussion”).

**DISCUSSION**

The spatial organization of the colonic mucosa implies that in vivo, cells normally undergo a sequence of events which includes proliferation, differentiation, apoptosis, and extrusion. In these studies we have shown that the cells which accumulate in the conditioned media of HT29 and SW620 human colonic carcinoma cell lines following standard, uninduced culture conditions are apoptotic, consistent with an earlier report based on morphological measurements (8). Furthermore, we have shown that these apoptotic cells exhibit increased ALP activity, an index of a more differentiated phenotype. Therefore, at least some of the cells in each of these two cell lines retain active pathways of differentiation and apoptosis and accumulate in the
linked to metabolic starvation resulting in mucosal surface degeneration and acute inflammation of the colonic epithelium (35, 37). Colonic instillation of SCFAs has been shown to be beneficial in reducing this inflammation and restoring homeostasis (37, 38).

Since SCFAs are the primary energy source of colonic epithelial cells and pivotal in maintaining homeostasis, the data showing that C4, but not isoC4 or fluoroC4, effectively potentiated differentiation and apoptosis suggest that the effects of SCFAs are linked to fatty acid structure and, most likely, utilization.

Unbranched, even numbered carbon atom SCFAs such as butyrate avidly undergo B-oxidation in the mitochondria, followed by entry of the generated acetyl-CoA into the citric acid cycle, and finally oxidative phosphorylation (58). On the other hand, branched isobutyric acid is converted into succinyl-CoA which enters the citric acid cycle and is used in the synthesis of glucose. Heptafuorobutyric acid, a fluorinated derivative of butyrate, is readily metabolized in the high fluorine-carbon bond energy (44, 45). Therefore, C4-potentiated differentiation, apoptosis, and accumulation of shed cells in the conditioned media of HT29 and SW620 colonic carcinoma cell lines may be specific to those SCFAs which, similar to butyrate, are not glucogenic but are fully oxidized in the mitochondria.

Epidemiological studies have linked fiber to the prevention of colon cancer but the mechanisms involved have not been resolved (59). Effects of fiber may, however, be complex. Poorly fermentable fiber may act by physical means such as affecting stool bulk and transit time (9). Fermentation of fiber, however, is the principle source of SCFAs from 2 to 4 carbon atoms long in the colonic lumen (9, 10). Thus, we have postulated that the protective effects of fiber may be linked to the generation of SCFAs and that their effects, in turn, on biochemical pathways associated with normal differentiation in colonic epithelial cells (25–28). The effect on apoptosis may be the end stage of these biochemical modulations and may be a mechanism by which fiber reduces the risk for tumor development (8).

Although the effects of butyrate on apoptosis represent only a 2–4-fold increase in a low level of spontaneous apoptosis (2–3%; Ref. 8 and data herein), they are statistically significant and, more important, biologically relevant. First, apoptosis is a rapid process and the phenotype is transient (2, 33, 34, 60). Second, the level of apoptosis detected in the adherent population is kept low by the fact that apoptotic cells detach and accumulate in the media (Ref. 8 and data herein). However, even these low rates are highly important. Bursch et al. (61) have demonstrated that a measurable level of approximately 3% apoptotic cells, similar to that present in this study, can result in tissue regression of 25% over several days if not balanced by proliferation. Finally, the increased apoptosis of 2–4-fold is clearly relevant in the context of potential SCFA effects on relative cancer risk. Epidemiologically, changes in relative risk of 2–3-fold associated with dietary exposures can be highly significant (62), and these alterations in relative risk are integrated values over decades of dietary exposure. Thus, the biochemical and cellular alterations in the mucosa brought about by dietary factors which affect relative risk in the general population are likely to be subtle.
Potentiation by Specific Short-Chain Fatty Acids of Differentiation and Apoptosis in Human Colonic Carcinoma Cell Lines

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