Steady-State Levels of Mitochondrial Messenger RNA Species Characterize a Predominant Pathway Culminating in Apoptosis and Shedding of HT29 Human Colonic Carcinoma Cells

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Abstract
A differentiated human colonic epithelial cell has undergone relatively stable molecular, biochemical, and cellular alterations resulting in the acquisition of structures, activities, and functions that characterize it as one of at least three mature phenotypes: a columnar absorptive, secretory, or enteroendocrine cell. We have shown previously that induction of HT29 cells with the short-chain fatty acid sodium butyrate elevates alkaline phosphatase activity, a marker of the absorptive cell phenotype, and increases mitochondrial gene expression. Furthermore, this induction is accompanied by subsequent apoptosis and cell shedding. In this report, we have investigated the effects of forskolin, a potent inducer of the MUC2 gene in HT29 cells, a marker of the secretory phenotype, and have shown that neither apoptosis nor mitochondrial gene expression are significantly stimulated. Thus, differentiation along the secretory cell lineage may not play a major role in apoptosis of colonic epithelial cells. Moreover, we have also investigated two polar solvents, DMSO and dimethylformamide, which have been reported to induce a more differentiated, but as yet not well characterized, phenotype in colonic carcinoma cells in culture. Although neither polar solvent induces alkaline phosphatase expression or MUC2 expression, both induce significant apoptosis, again associated with significant elevation of mitochondrial gene expression. Thus, elevation of mitochondrial gene expression appears to be an important pathway in the induction of apoptosis in colonic epithelial cells in culture, whether or not markers characteristic of differentiation along either the absorptive or secretory cell lineage are induced.

Introduction
In the normal colonic mucosa, cell proliferation takes place in the lower regions of the colonic crypt and is extensive, generating approximately \(10^7\) cells every hour in the adult (1). To balance this proliferation and thereby maintain the number of cells populating a crypt, cells undergo apoptotic cell death, primarily in the upper regions of the crypt, the area of terminal differentiation, and extrusion into the colonic lumen (2–4). The events encompassing proliferation through cellular extrusion are rapid; nearly the entire colonic mucosa is replaced every 6 days (5). Thus, differentiation, apoptosis, and extrusion must be tightly balanced, relying on spatially and temporally linked genetic programs and exogenous stimuli.

As a result of genetic alterations associated with tumorigenesis, normal colonic epithelial cell differentiation is blocked or delayed, and the proliferative region expands (5, 6). Consequently, apoptosis and cellular extrusion may be delayed as well, providing the retained abnormal cells the opportunity of accumulating additional genetic alterations that further enhance their growth advantage (7, 8), the paradigm of tumor development and progression in the colon (9).

Our previous work has suggested that alterations in mt expressions are early events in colonic tumorigenesis. On the one hand, mt gene expression is depressed in the flat mucosa of patients at high genetic risk for developing colon cancer, and expression progressively decreases compared to normal colonic mucosa during tumor development (10–12). These findings may be directly linked to the alterations in mt structure and function that are characteristic of colonic tumors (13, 14). On the other hand, unbranched, metabolizable SCFAs induce colonic carcinoma cells to differentiate in vitro, generating cells that express a marker characteristic of the luminal absorptive lineage, i.e., ALP. These cells also exhibit increased levels of mt steady-state mRNA species, increased mt enzymatic activity (10, 15), and undergo enhanced apoptosis and cellular shedding (16, 17).

Received 8/30/95; accepted 10/31/95.

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1 This work was supported in part National Cancer Institute Grants R29 CA59932, R01 CA56858, and PO CA1330 and American Institute for Cancer Research Grant 94A25.

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In this study, we addressed the question of whether forskolin induction of the MUC2 gene, which encodes the major colon mucin backbone, a product characteristic of the secretory cell lineage, is also linked to apoptosis of HT29 cells, and furthermore, whether this pathway is associated with elevated mitochondrial gene expression. Finally, we investigated whether two polar solvents, DMSO and dimethylformamide, which have been reported to induce a more differentiated, but as yet not well-characterized, phenotype of colon carcinoma cells, were also capable of inducing apoptosis and mitochondrial gene expression. The results establish that pathways of differentiation linked to induction of the MUC2 gene do not result in increased mitochondrial gene expression, apoptosis, or cell shedding. Moreover, these and our previous studies demonstrate that enhanced apoptosis is associated with those pathways in which elevated mitochondrial gene expression is a component.

**Results**

*In vivo*, the genetic program governing colonic epithelial cell differentiation is likely to be modulated by multiple homo- and heterotypic cell interactions, growth factors, and macronutrients. This is reflected in the fact that a large variety of agents can modulate aspects of colonic epithelial cell differentiation *in vitro*. Among these are SCFAs (7, 15, 17–19), polar solvents (20–22), activators of signal transduction pathways (23), nonlabile glucose derivatives (24), and glucose deprivation (25, 26).

Induction of HT29 colonic carcinoma cells with the SCFA butyrate increases ALP activity, a product characteristic of the absorptive cell lineage (7, 17), and concomitantly elevates steady-state mitochondrial mRNA levels (10, 15), apoptosis, and cell shedding (7, 16). Therefore, we asked whether forskolin, which has been demonstrated to enhance colonic mucin gene expression, a characteristic of the secretory cell phenotype (23), would also stimulate mitochondrial gene expression, apoptosis, and cell shedding.

Consistent with this previous work, we found that forskolin induction significantly increased steady-state levels of MUC2 mRNA, the major colonic mucin protein backbone (Fig. 1, □; *P < 0.01). However, as also shown previously (27), neither steady-state mRNA levels of ALP (Fig. 1) nor enzymatic activity levels of ALP (data not shown) were induced by forskolin. Forskolin induction was also ineffective in altering steady-state levels of the mt genes COI, COIII, and ND6 (Fig. 1). We also found that forskolin does not induce significant levels of apoptosis, as determined by the per centage of fragmented DNA to oligosomal size fragments (Fig. 2; *P > 0.05). Consistent with this low level of apoptosis, there was no increase in the number of cells shed in response to forskolin induction (Fig. 2).

Polar solvents have been shown to modulate several aspects of colonic cell growth and tumorigenesis. *In vitro* exposure of colonic carcinoma cells to the polar, planar solvents DMF or DMSO reduces growth rates and clonogenicity in soft agar (20, 21), increases the number of desmosomes (22), and decreases tumorigenicity in nude mice (20), alterations consistent with differentiation to a more normal phenotype. However, markers typical of the established colonic epithelial cell lineages have not, as yet, been identified following induction with these polar solvents. In these studies, we investigated the effects of polar solvents on levels of ALP.
gene expression and activity, MUC2 expression, and mitochondrial gene expression, apoptosis, and cell shedding.

Neither DMF nor DMSO affected steady-state levels of ALP mRNA (Fig. 1; ■ and ■, respectively) or ALP enzymatic activity (data not shown). However, induction with either polar solvent significantly decreased steady-state MUC2 mRNA to levels below those of uninduced, control cells (Fig. 1). The data of Fig. 1 are steady-state levels at 72 h of continuous exposure to the agents. To rule out the possibility that DMF or DMSO induced either ALP or MUC2 expression transiently at an early time point, an additional experiment was done following 24 h of exposure. Table 1 illustrates that expression of neither marker was elevated by DMF or DMSO at this time point, and in fact, that steady-state levels of MUC2 mRNA were already decreased by 24 h. Thus, although DMF and DMSO have been reported to induce a more differentiated and less tumorigenic colonic cell phenotype, they do not elevate these markers that characterize either the absorptive or the secretory cell lineage. However, similar to SCFA-induced differentiation, both polar solvents significantly elevated steady-state levels of mt genes (COI, COIII, and ND6; Fig. 1). Further, both polar solvents also induced highly significant increases in apoptosis, as measured by DNA fragmentation, and cell shedding (P < 0.01; Fig. 2).

Apoptosis was also evaluated in these experiments by PI staining and flow cytometry. Using this method, apoptotic nuclei appear as a broad hypodiploid DNA peak that is readily discriminated from the narrow peak of nuclei containing diploid DNA (28). Fig. 3 shows two representative profiles that illustrate the striking contrast between profiles from diploid and hypodiploid nuclei. The upper profile is from uninduced adherent cells and shows the sharp peak of PI-stained nuclei typical of cells containing diploid DNA (Fig. 3, arrow). The lower profile, from uninduced shed cells, represents staining of a population of cells that have undergone, or are undergoing, extensive apoptosis (7). The pronounced broad DNA peak extending to the left of the diploid peak (Fig. 3, arrow) is characteristic of hypodiploid nuclei from apoptotic cells. These extensive hypodiploid peaks were seen in shed cell populations regardless of induction (data not shown). Characteristic small hypodiploid peaks were also evident in the profiles from PI-stained DMF- and DMSO-induced adherent cells, while a minimal peak of hypodiploid nuclei was seen in adherent cells following induction with forskolin (data not shown). These peaks were quantified, and regression analysis was used to compare the percentage of apoptotic nuclei in adherent cells determined by PI staining and flow cytometry with the percentage of fragmented DNA in adherent cells determined by the DPA reaction. As illustrated in Fig. 4, quantitation of apoptosis by these two methods shows excellent correlation, thus confirming and corroborating the minimal apoptosis induced by forskolin and the more extensive, significant apoptosis mediated by DMSO or DMF induction of HT29 cells.

Results from our previous work on SCFA induction (7, 15), combined with our results from forskolin-, DMF-, or DMSO-induced HT29 cells presented here are summarized in Table 2. These data demonstrate that not all pathways of differentiation trigger apoptosis and consequent cell shedding, since significant induction of MUC2 expression does not result in
Discussion

Our previous work suggested that apoptosis and cell shedding were associated with the columnar absorptive cell lineage (7) and were characterized by up-regulation of mitochondrial gene expression and mitochondrial enzymatic activity (15), which are depressed in colonic tumors (10). In this report, we continued to dissect the complex relationships among lineages of differentiation, pathways within those lineages, apoptosis, and cell shedding in HT29 cells. Cells induced by forskolin, which up-regulates expression of the MUC2 and MUC3 genes, markers of the colonic secretory cell phenotype (27, 29), were found to have unaltered steady-state levels of mt gene expression and to undergo minimal apoptosis and shedding. There is additional evidence that differentiation along the secretory lineage results in, at most, minimal apoptosis of the bulk of the induced cells. A subclone of HT29 cells, referred to as 16E cells, differentiate upon confluence into highly polarized, nontumorigenic goblet cells (27, 30); replete with the elaboration and secretion of glycosylated colonic mucin (30). Neverthe-

less, although virtually all of the cells are well-differentiated goblet cells (27, 30), they do not undergo extensive apoptosis and, upon re-seeding at lower densities, revert to an undifferentiated, highly malignant phenotype of rapidly growing cells (30). Furthermore, since apoptosis is the end point of at least some colonic epithelial cell differentiation pathways, a pathway that results in apoptotic cell death should minimize proliferative potential. Flow cytometry data suggest that at least some of the cells in the forskolin-induced HT29 population may enter the cell cycle. Moreover, goblet cells in vivo retain proliferative potential during the process of mucin production (31). Thus, those cells that differentiate along the secretory cell phenotype may not contribute significantly to the cell population that undergoes apoptosis in the colonic mucosa.

Although we found that induction with the polar solvents DMF or DMSO does not generate cells that express established markers of either the secretory or columnar absorptive lineages, steady-state mitochondrial mRNA levels are increased, and apoptosis and cell shedding are potentiated. Thus, there is a consistent link between the induction of mt gene expression and subsequent apoptosis in culture. However, this link between mt, mfn function, and apoptosis is likely to be complex. There are several reports consistent with our data that suggest a role for mitochondrial function in apoptosis. Newmeyer et al. (32) recently described a system in which morphological nuclear alterations resembling those of cells undergoing apoptosis are reproduced when nuclei are added to Xenopus egg extracts. In this system, apoptosis occurred only when a cellular fraction enriched in mt was added. Inhibition of mt function or the addition of exogenous BCL2 protein delayed apoptosis. Alterations in mt function, reflected in changes in the mitochondrial membrane potential, have also been linked to apoptosis in other systems. Transfection of bcl2 into L929 mouse fibrosarcoma cells increased mitochondrial membrane potential, and the cells became resistant to tumor necrosis factor-induced apoptosis (33). Similarly, decreased mitochondrial membrane potential and uncoupling from oxidative phosphorylation were linked to apoptosis induced by growth at the nonpermissive temperature of rat embryo fibroblasts immortalized with a temperature-sensitive mutant of SV40 large T antigen (34). On the other hand, Jacobson et al. (35) demonstrated that a human fibroblast cell line immortalized by SV40 large T antigen and lacking mitochondrial DNA can still be induced to undergo a bcl2-sensitive apoptosis by direct and indirect deprivation of external survival factors. In a subsequent report, we demonstrate that part of the resolution for these conflicting data may be that mitochondrial function is linked to the commitment of colonic carcinoma cells to apoptosis but is not necessary for the process of apoptosis.

Finally, it is likely that mitochondrial function is linked to apoptosis in the process of colon tumorigenesis in vivo. Accumulation of genetic alterations that directly or indirectly interfere with normal differentiation is the paradigm of tumor development in the colon (9). During this process of tumor-

Table 2 Effects of induction of HT29 cells with different agents

<table>
<thead>
<tr>
<th>Inducer</th>
<th>ALP</th>
<th>Mt exp</th>
<th>MUC2 exp</th>
<th>Apop.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCFAs*</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>Forskolin</td>
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<tr>
<td>DMF</td>
<td>↓</td>
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</tr>
<tr>
<td>DMSO</td>
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</tbody>
</table>

* From Refs. 7 and 15.
†, significant increase (P < 0.05); ↓, significant decrease (P < 0.05); –, no effect.
igenesis, mitochondrial gene expression progressively decreases (10), and depressed mitochondrial enzymatic activity (13) and increased mitochondrial membrane potential (36–38) are characteristic of colonic tumors. This is associated with a progressive decrease in the number of apoptotic cells during colonic tumor development until, in frank carcinoma, very few apoptotic cells are detected (4). Thus, in vivo, the alterations in mitochondrial gene expression and function in colonic tumors most likely disrupt a pathway that consequently affects commitment of the cells to undergo apoptosis.

Materials and Methods

Induction of Cells with Polar Solvents or Forskolin

The HT29 human colonic carcinoma cell line, originally established by Fogh and Trempe (39), was obtained from ATCC and maintained as described previously (15). Because HT29 cells are able to assume a more differentiated phenotype coincident with glucose deprivation (25, 26), confluent cultures were fed 24 h before induction for 72 h with fresh media containing DME (Sigma Chemical Co.) at 1% (v/v), DMSO (Sigma) at 2% (v/v), or forskolin (Sigma) at 10 μg/ml ethanol. Cells that accumulated in the conditioned media during the induction period were harvested by centrifugation, and adherent cells were obtained from the same flask by scraping (7).

Apoptosis

Apoptosis was evaluated by two methods.

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 amount of DNA in the sample (40, 41). In brief, adherent and shed cell pellets were resuspended in hypotonic lysis buffer (10 mm Tris, 1 mm EDTA (pH 7.4), and 0.02% Triton X-100) and held on ice for 10 min. The lysates were centrifuged at 13,000 × g for 10 min, and both the pellet (containing the high molecular weight DNA) and the supernatant (containing the low molecular weight DNA) were precipitated at 4°C with 12.5% trichloroacetic acid. After centrifugation (13,000 × g for 10 min), precipitated DNA was extracted into 80 μl of 5% trichloroacetic acid by heating at 90°C for 10 min. Perchloric acid was added to a final concentration of 0.5 N.

DNA concentration was determined by the DPA reaction. Two hundred μl of freshly prepared DPA reagent (150 mg DPA; Sigma), 150 μl H2SO4, and 50 μl of acetaldehyde (at 16 mg/ml) of glacial acetic acid were added to each of the extracted DNA samples, and the reaction was allowed to develop at 30°C overnight. Aliquots (140 μl of the reaction were transferred to flat-bottomed polystyrene plates, and the absorbance at 570 nm was measured using a Model 450 microplate reader (Bio-Rad). A DNA standard curve was generated using sheared salmon testes DNA. Data are expressed relative to uninduced control.

PI Staining. The method of Niccolletti et al. (28) was used to measure apoptosis by PI staining and flow cytometry. Briefly, adherent and nonadherent cells from uninduced and induced cultures were gently pelleted, resuspended in hypotonic fluorochromic solution [50 μg/ml propidium iodide (Sigma) in 0.1% sodium citrate plus 0.1% Triton X-100] and held in the dark at 4°C overnight. The PI fluorescence of individual nuclei (10,000 events) was determined using a FACScan flow cytometer (Becton Dickinson) as described (28).

Cellular Shedding

Cells which were shed into the conditioned media following induced or uninduced culture conditions were harvested by centrifugation. DNA was extracted and quantified as described above. Total DNA/ml of conditioned media was determined as an index of cell shedding (7). Data are expressed relative to uninduced control.

RNA Isolation and Analysis

RNA was isolated from adherent control and induced cells using cesium cushions as described (15). The following probes were purified inserts labeled with [32P]dCTP by the random priming method (42): glyceraldehyde-3-phosphate dehydrogenase, pHógAPR NR (ATCC 57091); placental-like ALP (ATCC 59651); COL 4–4P (a generous gift from L.B. Chen, Harvard Medical School, Boston, MA); COL11, p50F1 (10); NDE6, 407 bp of the published human mitochondrial sequence NDE6 (43), amplified using PCR, cloned, excised and purified; and MUC2 (C22b), a generous gift from A. Velcich (Albert Einstein Medical School, Bronx, NY; Ref. 23).

Quantitation of Steady-State mRNA Levels. Steady-state mRNA levels were quantified by high stringency hybridization to replicate dot blots as we have described (15). Data are expressed relative to uninduced control.

Statistical Analyses

At least three individual determinations of % fragmentation, cellular shedding, and steady-state mRNA levels were made. Two-sample t tests using individual groups, pooled variances, were done using GB-Stat computer-aided statistics, version 1.0 (Dynamic Microsystems). P values of <0.01 were considered significant.

References


