Short Chain Fatty Acids and Colon Cancer\textsuperscript{1,2}

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ABSTRACT The development of intestinal cancer involves complex genetic and epigenetic alterations in the intestinal mucosa. The principal signaling pathway responsible for the initiation of tumor formation, the APC–\(\beta\)-catenin–TCF4 pathway, regulates both cell proliferation and colonic cell differentiation, but many other intrinsic and extrinsic signals also modulate these cell maturation pathways. The challenge is to understand how signaling and cell maturation are also modulated by nutritional agents. Through gene expression profiling, we have gained insight into the mechanisms by which short chain fatty acids regulate these pathways and the differences in response of gene programs, and of the specific regulation of the \(c\)-\textit{myc} gene, to physiological regulators of intestinal cell maturation, such as butyrate, compared with pharmacological regulators such as the nonsteroidal antiinflammatory drug sulindac. Moreover, we used a combination of gene expression profiling of the response of cells in culture to sulindac and the response of the human mucosa in subjects treated with sulindac for 1 month, coupled with a mouse genetic model approach, to identify the cyclin dependent kinase inhibitor \(p21\text{WAF1/Cip1}\) as an important suppressor of Apc-initiated intestinal tumor formation and a necessary component for tumor inhibition by sulindac. Finally, the mucus barrier, secreted by intestinal goblet cells, is the interface between the luminal contents and the intestinal mucosa. We generated a mouse genetic model with a targeted inactivation of the \(\text{Muc2}\) gene that encodes the major intestinal mucin. These mice have no recognizable goblet cells due to the failure of cells to synthesize and store mucin. This leads to perturbations in intestinal crypt architecture, increased cellular proliferation and rates of cell migration, decreased apoptosis and development of adenomas and adenocarcinomas in the small and large intestine and the rectum.


KEY WORDS: intestinal cancer • gene expression profiling • mouse genetic models • mucin • cell maturation

Nutritional factors play a major role in determining the probability of development of most sporadic colorectal cancer (1). The challenge we face is identification of the mechanisms through which nutritional factors perturb fundamental pathways that establish and maintain intestinal homeostasis and how these alterations modulate the likelihood of tumor initiation and progression. The intestine is a continuously regenerating epithelium. Stem cells, still undefined, reside near the bottom of the crypt. These cells, and their progenitors, undergo cell division, but the daughter cells eventually cease dividing after they have migrated about two thirds of the distance up the crypt. This limitation on proliferative potential appears to be due to an integration of external signals, from the contents of the intestinal lumen, with genetic programs that are activated to generate intracellular and intercellular signals that regulate not only proliferation but other aspects of cell maturation as well—lineage-specific differentiation and apoptosis.

An important pathway that regulates at least some aspects of these developmental processes was discovered as a result of its role in initiating the development of most human colorectal cancer. Mutations in the \(\text{APC}\) gene are responsible for the disease familial polyposis (FAP),\textsuperscript{4} a syndrome in which patients develop hundreds to thousands of benign tumors of the colon, some of which will progress to cancer if not removed. In sporadic colon cancer, mutations in \(\text{APC}\)—or, more rarely, alterations in a pathway that it regulates—initiate the development of almost all tumors (2). The difference in frequency of tumors between sporadic disease (one tumor over six to seven decades of life) and FAP (hundreds of tumors by the age of 20–30 y) can be attributed at least in part to the fact that tumor development requires the inactivation or loss of both \(\text{APC}\) alleles and, in FAP patients, the first event is present at birth.

The \(\text{APC}\) gene encodes a protein of \(\sim 300\text{ kDa}\) with mul-

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\textsuperscript{4}Abbreviations used: FAP, familial polyposis; SCFA, short chain fatty acid.
tiple functional domains (3). However, it is the role of APC in regulating β-catenin–TCF, or Wnt, signaling that has achieved the most attention thus far as a pathway through which tumor formation can be initiated. The APC protein interacts in a complex with β-catenin, glycogen synthase kinase 3β and axin to regulate the levels of β-catenin by targeting β-catenin for degradation by the ubiquitination-proteosome pathway. In the absence of functional APC, β-catenin levels rise, enabling it to form an active complex with the transcription factor TCF-4 (3). The β-catenin–TCF-4 transcription complex targets the expression of several important genes, including cyclin D1 (4,5) and c-myc (6), the latter in turn activating the transcription of the cyclin-dependent kinase, cdk4 (7). This activation of the cell cycle machinery brings about the increase in cell proliferation and expansion of the proliferative compartment that is seen in subjects at high risk for the development of colon cancer (e.g., Ref. 8). The importance of the pathway in tumorigenesis is emphasized by the fact that mice in which there is an inherited inactivation of APC also develop intestinal tumors linked to an increase in mucosal cell proliferation (9,10).

The β-catenin–TCF pathway and differentiation

To investigate the role of the β-catenin–TCF pathway in the intestine, Clevers and colleagues generated a mouse with a targeted inactivation of the key transcription factor TCF-4 (11). These animals die soon after birth, and the mechanism of this lethality is instructive. Stem cells at the base of the crypt in the small intestine differentiate. Therefore, the intestinal epithelium cannot be maintained, and the animals bleed to death. This was evidence that β-catenin–TCF signaling might also regulate aspects of cell differentiation. We used the Caco-2 cell line as a model system to investigate this. As a function of time in culture, Caco-2 cells undergo contact inhibition of cell proliferation and differentiate along the absorptive cell lineage. We demonstrated that this was coincident with a down regulation in β-catenin–TCF complex formation and activity, consistent with the decrease in cell proliferation (12). We also demonstrated that the expression of four genes that characterize the absorptive cell phenotype (alkaline phosphatase, sucrase-isomaltase, carboxyembryonic antigen and intestinal fatty acid binding protein) was elevated due to increased activity of their promoters with time in culture. To determine if this was linked to β-catenin–TCF signaling, we down-regulated the β-catenin–TCF pathway in undifferentiated Caco-2 cells by introducing any one of three different expression vectors into the cells: wild-type APC, which led to targeting of cytoplasmic β-catenin for degradation; E-cadherin, which, when overexpressed, sequestered β-catenin at the cell membrane, preventing formation of a complex with TCF; and a dominant-negative TCF-4, which inhibited activity of the β-catenin–TCF transcription complex. All three expression vectors, as expected, down-regulated β-catenin–TCF signaling. In addition, all three reduced the promoter activity of cyclin D1, as well as increased the activity of the promoters for alkaline phosphatase and intestinal fatty acid binding protein, although none of the expression vectors affected the promoter activities of CEA or sucrase-isomaltase (12).

There are two important conclusions from these observations: first, β-catenin–TCF signaling is linked to differentiation of intestinal epithelial cells; and second, this must be complex, because the promoters of only two of the four genes studied showed response. This complexity was not unexpected. There are a large number of signals impinging on cells in the intestinal mucosa, derived from cell–cell and cell–matrix interactions, as well as contact of the cells with the luminal contents. More specifically, we had already shown that the short chain fatty acid (SCFA) butyrate, a physiological inducer of intestinal cell maturation that is derived from fermentation of fiber, and that is present at high concentration in the lumen, could stimulate aspects of the absorptive cell phenotype in Caco-2 cells that were not stimulated by contact inhibition of growth (13).

Early gene expression profiling: defining the role of mitochondria

We have had a long interest in developing and using methods for investigating such complexity in developmental and disease processes. In the early 1980s, we used arrays of 400 sequences to characterize a chemically induced mouse colon tumor (14) and, by the mid-1980s, had developed the first computerized scanning and image processing system for quantitative analysis of each of 4,000 sequences in cells in culture, as well as in mucosal and tumor biopsy samples (15). Using this system, we identified a panel of genes that distinguished the flat intestinal mucosa at risk for tumor development from low-risk mucosa (16). This was the first example of profiling of gene expression, especially to define clinical phenotype (16,17).

These investigations led to our understanding that the mitochondria, the site of metabolism by β-oxidation of SCFAs, was intimately involved in regulating—and, more important, coordinating—cell proliferation and apoptotic pathways in the intestinal mucosa (18–23). The data from a number of our investigations suggested that this metabolism was a necessary component of at least some of the responses to the SCFA butyrate. This was pursued by using a mouse with a homozygous deletion of the gene for short chain acyl dehydrogenase, which encodes an enzyme necessary for the mitochondrial β-oxidation of SCFAs. In these mice, we found that elimination of the ability to efficiently metabolize SCFAs reduced the level of apoptosis in the colon by >90% (24). Interestingly, however, there was no reduction in apoptosis in the small intestine. Because SCFAs are formed by microbial fermentation of fiber in the large intestine, we interpreted the results to suggest that cells in the large intestine had adapted, or evolved, pathways that linked their normal processes of maturation to their physiological utilization of SCFAs as an energy source. In contrast, cells in the small intestine, not being exposed to the same high levels of SCFAs, do not use these pathways in the same way and are not dependent on them for their normal maturation. More recently, we have shown, and discussed, that the mitochondrial membrane potential may be the key factor through which maturation pathways are regulated by SCFAs (23,25). We have also found that the intrinsic mitochondrial membrane potential has a significant impact on the growth properties and response of colonic carcinoma cells to various chemopreventive and chemotherapeutic agents (B. G. Heerdt and M. A. Houston, unpublished).

Microarray analysis of gene expression and transcriptional imaging

To investigate this response to the SCFA butyrate in greater detail, we have used microarray analysis of the expression of >8,000 sequences in the response of the SW620 colon carcinoma cell line to butyrate and compared this with three other inducers of maturation: trichostatin A, like butyrate, an
inhibitor of histone deacetylase activity; sulindac, a nonsteroidal anti-inflammatory drug that is an effective inhibitor of intestinal adenoma formation in FAP patients; and curcumin, a component of curry and mustard that has been shown to have chemopreventive activity. In response to butyrate, the cells show alterations in gene expression that expand continuously as a function of time over 48 h after exposure (26).

Although the cells also showed changes in expression in response to the other agents, this monotonic expansion was not seen. We believe that such a pattern of response is characteristic of a cascade of preprogrammed events. That is, in response to butyrate, the cells activate a program of alteration of gene expression, with each stage an amplification of the response triggered by the previous stage. The fact that such a pattern is seen only with the physiological inducer butyrate is a second example that suggests that cells have evolved to respond in a programmed way to compounds which they usually encounter, as opposed to pharmacological agents to which they have not been previously exposed. We will return to this in considering the regulation of the c-myc gene.

In addition to the differences in patterns of altered gene expression as a function of time, it was also clear that the genes recruited into the response by the various agents were different (26). This was true not only for the overall gene set but also for functional classes of genes involved in signaling pathways or in cell cycle regulation and progression. This was surprising because butyrate, sulindac and trichostatin A all seemed to cause the same G0/G1 cell cycle arrest, although with curcumin, the arrest was in G2/M. Thus, we concluded that the response of the cells could not be predicted based only on the expression of one or several genes but that the response was due to the integrated effects of a large number of genes.

One gene in particular presented an interesting pattern of response. c-myc expression was decreased in response to butyrate. This decrease in c-myc in response to butyrate was well-established in the literature and was consistent with the G0/G1 cell cycle arrest stimulated by butyrate but was inconsistent with the fact that we had shown previously that β-catenin-TCF complex formation and activity, which directly target c-myc, was increased by butyrate (27). Moreover, in our array analysis, sulindac, which also produced a G0/G1 cell cycle arrest, instead elevated c-myc expression. We therefore investigated the expression of c-myc by more incisive methods. This involved identifying c-myc transcription sites within the nuclei of cells, with probes that recognize either the 5′ end of the message, or the 3′ end, to distinguish transcription sites that were initiated (only detected by 5′ probes) from those that were initiated and completed (detected with both 5′ and 3′ probes) (Wilson et al., Cancer Res. in press). Using this method, we found that both butyrate and sulindac increased the number of cells with detectable c-myc transcription sites, consistent with our previous report that both elevate β-catenin-TCF activity. However, in response to butyrate, a transcriptional pause mechanism near the intron 1/exon 1 border was recruited, so that many of these initiated transcripts were not completed. In contrast, the pause mechanism was not recruited by sulindac. The consequence was that although both agents activate initiation of transcription of the c-myc gene, in response to butyrate, the block to transcription led to lower levels of c-myc, whereas in response to sulindac, c-myc levels rose. We believe that this is a specific example of cells having evolved mechanisms to fine-tune their response to exogenous agents that they normally encounter and to integrate this response with their physiology. In contrast, this is absent for new pharmacological agents. This has important implications: the block to c-myc transcription recruited in response to butyrate leads to lower expression of the gene, consistent with the cell cycle arrest induced. However, the elevation of c-myc in response to sulindac is incompatible with the cell cycle arrest. Because elevated c-myc in cells that are otherwise arrested in the cell cycle leads to apoptosis (28,29), this may compound the toxic side effects associated with long-term use of sulindac, such as ulceration and bleeding, that have been attributed to the inhibition of cyclooxygenase I activity.

**Genomic and genetic analyses of the response to sulindac**

Although sulindac has serious side effects that may limit its clinical use, it is an effective chemoprotective agent. Consequently, it is important to understand its mechanism of action, so that we may capitalize on its efficacy while minimizing its toxicity. We therefore pursued microarray analysis of gene expression using rectal biopsies of subjects taken before, and after, 1 mo of daily sulindac treatment. Complex and heterogeneous changes in gene expression were detected in subjects. This database was then compared with the data generated from treatment of SW620 cells in culture with sulindac. We first identified a set of sequences that were down-regulated by sulindac in vivo but either not expressed, or not altered in expression, in tissue culture (30). These were all sequences normally expressed in lymphocytes. This was interesting because sulindac, an anti-inflammatory drug, would be expected to reduce lymphocyte invasion in the mucosal biopsy samples but not in the pure population of epithelial cells in culture. Therefore, this observation was consistent with the fact that the microarray analysis accurately reflected the profile of gene expression in the tissue.

A second subset of sequences identified were those that were altered similarly by sulindac both in vivo and in tissue culture (30). Only eight sequences—0.1% of the total investigated—satisfied these criteria. One of particular interest was the cyclin kinase–dependent inhibitor (cdki) p21 WAF1/cip1. This was interesting because p21 had been shown to be up-regulated in response to sulindac in vitro, which was consistent with the cell cycle arrest that was induced. Our microarray data extended this to the intestinal mucosa. To determine whether this up-regulation of p21 was only a marker of response to sulindac or was functionally significant, we generated a mouse model. A mouse with a targeted inactivation of p21 (homozygous) was crossed with the Apc1638/ mouse (10) with the p21 WAF1/cip1 knock-in model. The animals did not have a pronounced phenotype and did not develop intestinal tumors (31). We reasoned that a role for p21 might only be evident in mice that also had a mutation that acted as an initiator of tumor formation. Accordingly, we crossed the Apc1638+/− mouse (10) with the p21−/− mouse (31) and examined mice that were in all cases Apc1638+/−, to initiate tumor formation, but either p21+/+ (wild-type), p21+/− (heterozygotes) or p21−/− ( homozygotes).

We found that inactivation of p21 increased Apc initiated intestinal tumor formation, decreasing animal life span, and that this effect was p21 gene dosage dependent (33). The increased tumor formation was linked to an increase in mucosal proliferation and a decrease in mucosal apoptosis and goblet cell differentiation. It had also been shown that a Western-style diet (high in fat and phosphate, low in calcium and vitamin D) could increase Apc-initiated tumor formation but that this was due to a promotional effect later in tumor formation (34). We therefore postulated that the Western diet and p21 inactivation would have independent, and therefore
additive effects on Apc-initiated tumors, and this was correct (33).

The critical experiment was then to determine how inactivation of p21 affected the response to sulindac. Consistent with the work of others, sulindac inhibited small intestinal tumor formation in mice that were Apc+/− and p21 wild-type (30). However, inactivation of a single p21 allele completely eliminated the tumor inhibition seen with sulindac (30). More recently, we have found that inactivation of one p21 allele dramatically reduced the ability of sulindac to induce expression of the remaining allele (W. C. Yang, unpublished). Therefore, the cdki p21 appears to play a critical role in suppressing tumor formation and in the ability of sulindac to inhibit tumorigenesis. It has recently been demonstrated that inactivation of another cdki, p27, may play a similar role, although this has not yet been investigated for the response to sulindac (35).

Mucins and colorectal cancer: a new mouse model

Although we have made reference to the exposure of the intestinal mucosal cells to the contents of the lumen, in fact there is a mucous barrier that coats, lubricates and protects the intestinal tract. This is synthesized as mucins by intestinal goblet cells and secreted by these cells as a thick, viscous mucous layer. It is striking that in aberrant crypt foci, preneoplastic lesions seen in subjects at risk for tumor development, in rodents treated with colon-specific carcinogens, and in the Apc1638 mouse, there is a depletion in goblet cells and in the mucin they produce (36). To determine whether this was functionally important in the development of tumors, we targeted the mouse Muc2 gene, which encodes the principal colonic mucin, for inactivation. This generated a Muc2-null mouse model, in which there was no compensatory overexpression of other mucin genes (37). The inactivation of Muc2 led to increased intestinal cell proliferation, decreased apoptosis and a profound increase in the rate of cell migration up the crypt. Most important, the mice developed adenomas and adenocarcinomas throughout the intestinal tract in the absence of any other chemical or genetic initiator of tumor formation (37).

Tumor formation in the Muc2−/− mice raises many questions. For instance, although there is an absence of Muc2 expression and no recognizable goblet cells, another marker of goblet cells, intestinal trefoil factor (Itf), is still expressed in the intestinal crypt with the same distribution as that seen in wild-type mice (37). Therefore, although goblet cells appear to be absent, the lineage may still be present even though the cells lack their characteristic shape due to their lack of mucin. It is therefore important to ascertain the extent to which the inactivation of a gene that encodes this terminal marker of differentiation perturbs other aspects of the lineage. This is important to understand why and how tumors form in these mice. Thus far, we know that tumor formation is coincident with elevated c-myc expression, but unlike tumors initiated by inactivation of Apc, the elevation in c-myc is not due to elevated β-catenin or an increase in its nuclear localization (37).

CONCLUSION

The architecture, number of cell types and diversity of signaling mechanisms and pathways in the intestinal mucosa provide a complex web of interactions that all contribute to normal mucosal function and maintenance of homeostasis. Thus, the number of ways in which nutritional factors can perturb this network is large. Fortunately, microarray analysis of gene expression and related methodologies in proteomics provide ways to address this complexity and focus light on key pathways that can be dissected by genetic and biochemical methods.

LITERATURE CITED


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