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Research Article

Regulation of β-catenin and connexin-43 expression: Targets for sphingolipids in colon cancer prevention

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Sphingolipid metabolites are generated throughout the intestinal tract after hydrolysis of orally administered complex sphingolipids and significantly suppress colon cancer in carcinogen-treated CF1 mice. In the present study, the mechanisms of tumor suppression by dietary sphingolipids were investigated. Changes in select genes that are critical in early stages of colon cancer were analyzed in the colonic mucosa of dimethylhydrazine-treated CF1 mice fed AIN76A diet with or without 0.05% sphingomyelin (SM). Supplementation with SM did not significantly alter mRNA levels of most of the selected genes. However, a downregulation of β-catenin ($p = 0.007$) and increased protein levels of connexin-43 ($p = 0.017$) and Bcl-2 ($p = 0.033$) were observed in SM-fed animals. This suggests that sphingolipids may be regulating specific post-transcriptional events to reverse aberrant expression of individual proteins. Since the dysregulation of β-catenin metabolism and its transcriptional activity in addition to a decreased intercellular communication has been causally linked to the development of colon cancer while a low Bcl-2 expression is associated with a worse prognosis in colon cancer, the reversal of these early changes may be important events in the prevention of colon cancer by orally administered sphingolipids, and may provide specific molecular biomarkers for sphingolipid efficacy *in vivo*.

Keywords: Bcl-2 / β-catenin / Colon cancer prevention / Connexin-43 / Sphingomyelin

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1 Introduction

Carcinogenesis is a complex multistage process resulting from gene mutations, deletions, translocations, and silencing which all lead to aberrant or dysfunctional gene expression. Although a single gene alteration may primarily be responsible for a specific cancer (*i. e.*, *Rb* in retinoblastoma, *p53* in Li-Fraumeni syndrome), in many other cancers, several causal genetic alterations have been identified. Many of these changes result in the altered life span of transformed cells, loss of cell cycle and apoptosis control and altered communication, adhesion, and/or migration properties. Colon cancer has been postulated to result from a series of multiple and consecutive genetic changes [1]. The propensity of these genetic changes for colon cancer is defined by the order and disease stage in which they occur. Neither *RAS* nor *p53* mutations both frequently seen in colon tumors can efficiently drive transformation [2], indicating that in the colon neither acts as the "gatekeeper", defined as a gene that has to be inactivated to disturb tissue homeostasis and allow for net proliferation [3]. In the colonic tissue, this gene has been identified as the *Adenomatous Polyposis Coli* (*APC*) gene, involved in the regulation of proliferation, migration, differentiation, and apoptosis of colonocytes. *APC* mutations have been found in 40–80% of sporadic colon cancer and in almost all cases of familial adenomatous polyposis [4, 5]. They already can be detected in aberrant crypt foci (ACF), which are one of the earliest visible morphological changes in colon carcinogenesis and putative colonic precursors of adenomas and adenocarcinomas

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Abbreviations: ACF, aberrant crypt foci; **APC,** *Adenomatous Polyposis Coli*; **DMH,** dimethylhydrazine; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **PKC,** protein kinase C; **SM,** sphingomyelin

in both rodents [6] and humans [7]. The resulting truncated APC gene product causes a dysregulation of β-catenin, a protein that not only connects E-cadherin to the cytoskeleton but also has important signaling functions in the Wnt pathway. Other early events in colon cancer progression include changes in proliferation, apoptosis, and cell – cell communication. The reversal of these early changes may prevent or decelerate tumor formation and, thus, represent promising targets for colon cancer prevention efforts.

Sphingolipids metabolites are lipid second messengers that are generated by growth factors, cellular stresses, or cytokines. Sphingolipid-mediated changes in proliferation, differentiation, survival, or cell death have been reported in many cell lines, and are specific for the cell type, the sphingolipid species that are generated, their concentration and subcellular localization. Bioactive metabolites are also generated by chemotherapeutic compounds such as taxol, cisplatin and daunorubicin, and natural chemopreventive agents (*i. e.*, resveratrol, EGCG, curcumin, and β-sitosterol) (see recent review [8]). Although there are numerous reports on the effects of sphingolipids and sphingolipid metabolites in cancer cells *in vitro*, fewer studies have been conducted to determine the effects of sphingolipids on cancer cells *in vivo*. One reason may be their inherent toxicity, rapid turnover, and their amphiphilic nature that make an organ-specific delivery problematic. Our previous studies were the first to show that complex sphingolipids administered orally significantly reduced carcinogen-induced ACF [9 – 12] and colon tumor formation in CF1 mice by up to 80% [13]. We have also shown the dietary sphingolipids suppressed tumor formation in Min mice, a mouse model for familial adenomatous polyposis which was associated with the regulation of β-catenin expression and localization [14]. Importantly, although the growth inhibitory/cytotoxic metabolites ceramide and sphingosine are released in the intestinal tract after hydrolysis of the complex sphingolipids (see a recent review [15] and [16]), no severe side effects by this route of administration were noted in any of our studies or by other laboratories [9 – 17]. In the present study we have characterized molecular changes in the colonic epithelium from carcinogen-treated CF1 after long-term exposure to orally administered sphingomyelin (SM) to gain insight into the underlying mechanisms of the observed ACF and tumor suppression. Here, we have focused on the specificity of changes in the expression of genes critical in the earliest stages of colon cancer. We investigated first changes in β-catenin which, following its dysregulation, has been causally correlated to the progression of early stages of colon cancer [2, 6] and is a target of dietary sphingolipids in Min mice [14]. To test the specificity of sphingolipid-mediated regulation of gene expression, we also examined the adhesion and communication proteins E-cadherin and Connexin-43. In contrast to the observed increase in β-catenin levels in early stages of colon cancer, the loss of the expression of these genes has been reported in early stages of cancer in many organs including the colon [18, 19]. Since orally administered sphingolipids reversed carcinogen-induced changes of cell proliferation and apoptosis rather than induced apoptosis [13], we also determined the effect of SM on the expression of a panel of pro- and anti-apoptotic proteins, and cell cycle regulators that have been shown to be targets of sphingolipids *in vitro*. Our studies reveal that sphingolipids regulate the expression of proteins closely associated with the earliest steps in colon carcinogenesis in a protein-specific manner which may be critical for the prevention of colon cancer by dietary sphingolipids.

2 Materials and methods

2.1 Tissue harvest

Female CF1 mice, 5 wks old from Charles Rivers Laboratories (Portage, MI) were injected i.p. with dimethylhydrazine (DMH) (30 mg/kg bodyweight, once *per* week for 6 wks). All mice were fed the semipurified, casein-based AIN 76A diet (see formulation [20]), which is essentially sphingolipid-free, throughout the study, but two groups received 0.05% SM supplements (by weight) either before (SM-early) or after tumor initiation (SM-late) to compare the preventive and therapeutic potential of SM. The SM was purified from a lipid whey extract as described previously [13]. Forty-five weeks after the last DMH injection, all mice were killed by CO_2 asphyxiation, the colons were excised, and the tumors measured and removed for separate analysis. The reduction of tumor incidence and the reversal of carcinogen-induced changes in proliferation and apoptosis in the sphingolipid-fed groups have been reported elsewhere [13].

Randomly selected colonic tissues without tumors were scraped gently with DNase and RNase-free microscopy slides to harvest the epithelial cell layer that was used for both mRNA analyses by RT-PCR and for Western Blot analyses of protein expression. Other colons were fixed in formalin, embedded into paraffin, and sectioned at 5 μm for immunohistochemical analysis.

2.2 Semiquantitative reverse transcription PCR (sqRT-PCR) assay of colonic mucosa scrapings

Total RNA was isolated from the epithelial cells (20 mg wet weight) using the RNeasy kit according to the manufacturer's instructions (Qiagen). Ten samples *per* group (control, SM-early, and SM-late) were processed individually; however, since there was no statistically significant difference in tumor incidence among the supplemented groups (both treatments with SM suppressed tumor incidence by approximately 80% [13]), the results from both SM-fed groups were consolidated for data analysis.

Table 1. Oligonucleotide primers for RT-PCR

Primers		Sequences	Product size (bp)
Actin	Forw	AGAGGGAAATCGTGCGTGAC	138
	Rev	CAATAGTGATGACCTGGCCGT	
GAPDH	Forw	TCACCACCATGGAGAAGGC	168
	Rev	GCTAAGCAGTTGGTGGTGCA	
PKG	Forw	CCTCCGCTTTCATGTAGAGGAAGA	366
	Rev	GTAAAGGCCATTCCACCACCAA	
Bax	Forw	ACCAAGAAGCTGAGCGAGTGTC	367
	Rev	ACAAAGATGGTCACGGTCTGCC	
Bad	Forw	AGGACTTATCAGCCGAAGCAG	738
	Rev	TTTCCTAAGGCCTCGAAAGAC	
Bcl-2	Forw	TGTGGCCCAGATAGGCACCCAG	370
	Rev	ACTTCGCCGAGATGTCCAGCCAG	
Bcl-XL	Forw	GACTGGTTGAGCCCATCTCTA	754
	Rev	GTGAGTGGACGGTCAGTGTCT	
E-cadherin	Forw	ACGTATCAGGGTCAAGTGCC	376
	Rev	CCTGACCCACACCAAAGTCT	
Connexin-43	Forw	CTGCCTTTCGCTGTAACACT	399
	Rev	CGCTCAAGCTGAACCCATA	
β-catenin	Forw	TTCGCCTTCACTATGGACTACC	559
	Rev	TTCGCCTTCACTATGGACTACC	
Cyclin D1	Forw	CTGGCCATGAACTACCTGGA	500
	Rev	GTCACACTTGATCACTCTGG	
c-myc	Forw	CCAGCAGCGACTCTGAGG	683
	Rev	CCAAGACGTTGTGTGTTC	
PKC bI	Forw	TGTGATGGAGTATGTGAACGGGGG	640
	Rev	TCGAAGTTGGAGGTGTCTCGCTTG	
PKC-bII	Forw	CATCTGGGATGGGGTGACAACC	420
	Rev	CGGTCGAAGTTTTCAGCGTTTC	

RNA was quantitated with the RiboGreen® quantitation Kit (Molecular Probes). A two-step procedure was used to amplify mRNA. First, cDNA was synthesized from 1.0 μg total RNA with 1 μL Oligo-dT primers using the ImProm II RT system (Promega). For the cDNA amplification, the PCR Master Mix Kit from Qiagen was used. Specific forward and reverse primers (Table 1) were added to the Master mix, heated to 94°C for 5 s, then cycled 40 times in a Gene Amplification PCR system (Applied Biosystems) at 94°C for 15 s melt, 60°C for 30 s annealing, and 68°C for 90 s extension. After 7 min at 68°C, the samples were cooled to 4°C. Duplicate reactions for the generation of cDNA as well as duplicate amplification of cDNA by PCR were routinely performed for the samples. Since we have observed in unrelated studies that changes in actin expression can occur during transformation, we included amplification of actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerol kinase (PGK) mRNA as housekeeping controls. The PCR products were examined after separation by electrophoresis on 0.8% agarose gels (BioRad) with an EagleEye UV detection system (Stratagen). Bands were densitometrically quantitated using Image J (NIH). Values were normalized for actin, GAPDH, and PGK expression.

2.2.1 Western blot analysis

Epithelial cell scrapings were lysed in RIPA buffer containing 1 mM NaF, 1 mM Na_3VO_4, and standard protease inhibitors (Roche) for 30 min on ice. The lysates were homogenized using 25 gauge needles, and cleared by centrifugation at 4°C for 30 min at 3000 × *g*. Protein (15 – 50 μg) were separated by SDS-PAGE using 10% gels. After transfer to PVDF membranes, the blots were blocked with 0.5% skim milk powder in TTBS, and probed with antibodies directed against β-catenin (Sigma), protein kinase C (PKC)-βI and II, cyclin D1, c-myc, Bax and Bcl-2 (from Santa Cruz), Connexin-43 (BD Biosciences), and E-cadherin (Zymed). β-Actin (Sigma) was used to normalize protein expression. The bands were incubated with horseradish peroxidase-conjugated secondary antibodies (BioRad), and visualized using ECL (Amersham). The bands were recorded onto X-ray film, measured densitometrically with Image J (NIH) and corrected for the expression of β-actin.

2.2.2 Immunohistochemical detection of proteins

The Western Blot results were confirmed by immunohistochemical staining using our standard procedures [14]. Briefly, sections were deparaffinized and rehydrated through graded alcohol. After steaming for antigen retrieval

and endogenous peroxidase blocking, the sections were blocked with 0.1% BSA and 0.1% calf serum in PBS, and incubated overnight with the primary antibodies. For β-catenin, we used the anti-β-catenin antibody from Santa Cruz. The ABC kit from Zymed followed by DAB treatment was used for visualization of the immunocomplex. The sections were briefly counterstained with hematoxylin (Zymed), dehydrated, cleared in xylene, and permanently mounted with Histomount® (Zymed). All images were digitally captured on a Nikon 80i epifluorescence microscope, equipped with DIC, and processed with Adobe Photoshop®.

2.2.3 Statistical analysis

Groups were compared using the unpaired *t*-test for groups that had values sampled from Gaussian distributions. Unpaired *t*-test followed by Welch's correction was used when the SDs differed significantly among the groups, and nonparametric Mann – Whitney test was used in groups that did not follow Gaussian distribution. All statistical analyses were performed with Instat 3.0a (GraphPad Software).

3 Results

3.1 Orally administered sphingomyelin regulates expression of β-catenin

Orally administered sphingolipids regulate β-catenin expression in an animal model for familial adenomatous polyposis [14]. To establish that β-catenin dysregulation is also an early event in carcinogen-induced colon cancer and to demonstrate that its regulation by orally administered sphingolipids is not limited to a specific animal model, the effect of the carcinogen treatment and orally administered SM on β-catenin expression and localization was determined by immunohistochemical analysis in the colons of CF1 mice. β-Catenin expression was very weak to moderate in mice not treated with the carcinogen (untreated controls) and was found to be strictly localized at the lateral membranes of cells lining the lumen of the colon (Figs. 1A, A′). β-Catenin expression was elevated after carcinogen treatment (Figs. 1B, B′) and, in addition to its localization in membranes, cytosolic stain was detected frequently. The cells of the intestinal lining were generally β-catenin positive, but moderate to intense β-catenin expression was also observed in colonic crypt cells. Feeding SM to carcinogen-treated mice reduced β-catenin expression (Figs. 1C, C′) and weak to moderate β-catenin levels were detected mostly in the membranes of cells lining the colonic lumen. Colonic tumors expressed high levels of β-catenin in the cytosol. Nuclear β-catenin was only detected in colon tumors (Figs. 1D, D′). This confirms that β-catenin dysregulation is evident also in carcinogen-treated CF1 mice, that this is an early event and that its regulation by dietary sphingolipids is not limited to a specific animal model.

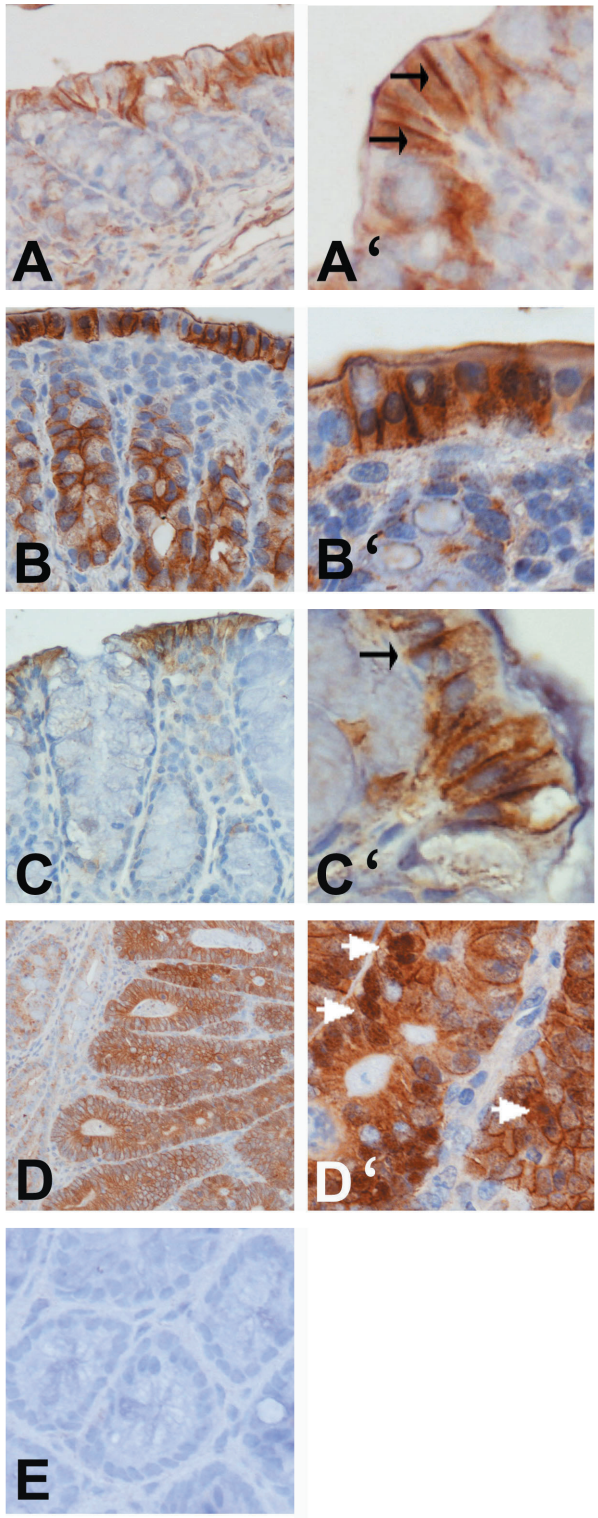

Figure 1. Changes of β-catenin expression. β-Catenin was determined by immunohistochemistry in the colon of untreated mice (A, A′), carcinogen-treated mice fed the control diet (B, B′), or SM supplements (C, C′). Nuclear β-catenin was only visible in the colon tumors (D, D′). Magnification 20× (A – D) or 40× (A′ – D′). E, antibody specificity control.

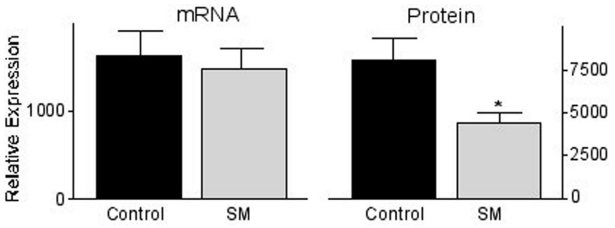

Figure 2. Sphingolipids downregulate β-catenin protein levels. Feeding 0.05% SM for 45 wks to carcinogen-treated CF1 mice has no effect on β-catenin mRNA levels in the colonic mucosa as determined by RT-PCR but significantly reduce its protein expression as determine by SDS-PAGE and Western Blotting. $^{*}p < 0.05$.

3.2 Sphingomyelin targets β-catenin protein levels rather than its transcription

Next, we investigated how sphingolipids regulate β-catenin levels *in vivo*. For these studies the colonic epithelium DMH-treated CF1 mice was used. DMH induces permanent transmissible alterations in macroscopically normal appearing colon cells and causes an increase in proliferation and a concomitant decrease in the rate of apoptosis [13, 21], both early events in human colon cancer and suggested driving forces for tumorigenesis. ACF formation could still be detected in the colons 45 wks after the last carcinogen injection [13], confirming that processes directing the colonic cells toward transformation and tumor development are constantly ongoing. Thus, the carcinogen-treated colonic mucosa comprises both macroscopically normal but primed cells and cells representing the earliest visible stages in colon carcinogenesis and therefore denotes the appropriate cell population for mechanistic prevention studies.

First, the mRNA expression levels of β-catenin in the colonic mucosa of carcinogen-treated CF1 mice were determined to evaluate an effect on β-catenin transcription. As shown in Fig. 2, there was no difference in β-catenin mRNA levels in colonic mucosa of mice fed the AIN 76A diet with or without SM supplements. Then, β-catenin protein expression was determined by Western Blot analysis. In contrast to the mRNA levels, β-catenin protein expression was significantly reduced in the dietary SM group ($p < 0.001$) (Fig. 2). These results confirm the immunohistochemical observations (see Fig. 1), and indicate a modulation of post-transcriptional or post-translational events by the sphingolipids resulting in lower protein expression rather than a regulation of β-catenin transcription. To determine if this is due to the regulation of processes that specifically modulate β-catenin expression or possibly the result of a general stimulation of protein metabolism/degradation that would result in an unspecific downregulation of both detrimental and beneficial proteins, the mRNA levels of connexin-43 and E-cadherin were also investigated. Both proteins are down-regulated in early colon cancer, and

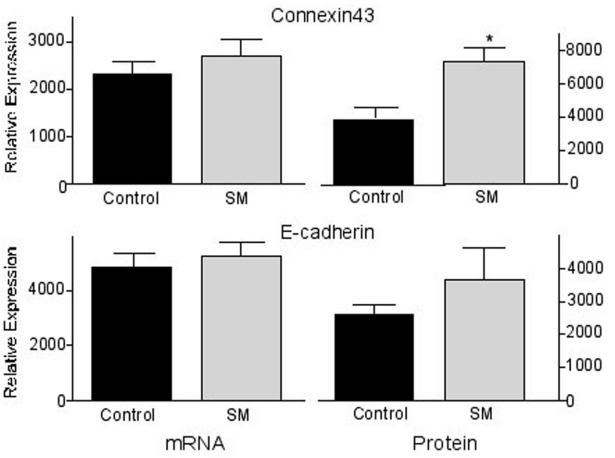

Figure 3. Sphingolipids upregulate E-cadherin and Connexin-43. SM supplements in the diet of carcinogen-treated CF1 mice upregulate connexin-43 and E-cadherin protein levels in the normal macroscopically appearing colonic mucosa (determined by SDS-PAGE and Western Blotting) without affecting their mRNA levels (determined by RT-PCR). $^{*}p = 0.017$.

Table 2. Changes in mRNA levels

	mRNA (relative expression, ± SEM)		
	Control ($n = 10$)	SM ($n = 20$)	p
Bax	4566.9 ± 381.8	5068.9 ± 354.6	0.3869
Bad	2146.4 ± 542.1	1725.9 ± 263.9	0.436
Bcl-2	2488.7 ± 508.2	3279.0 ± 386.3	0.237
Bcl-XL	2882.1 ± 795.4	2534.4 ± 519.5	0.710
Cyclin D1	1426.0 ± 445.8	993.4 ± 192.8	0.305
c-myc	1599.5 ± 451.0	1801.4 ± 312.4	0.714
PKCβI	**152.8 ± 62.5**	**712.9 ± 163.1**	**0.025**
PKCβII	**2035.8 ± 242.9**	**3374.9 ± 408.4**	**0.036**

thought to be critical for tumor development since the restoration of expression can reverse the phenotype of the cells [22 – 24]. As shown in Fig. 3, there was no significant effect of the dietary SM on mRNA levels of either protein; this is comparable to what has been seen with β-catenin mRNA levels. However, in contrast to our results with β-catenin protein expression, there was an elevation in protein levels of both E-cadherin and connexin-43 after sphingolipid feeding ($p = 0.017$ for connexin-43; the increase in E-cadherin expression did not reach statistical significance) (Fig. 3).

3.3 Sphingolipids do not change the expression of targets of β-catenin-mediated transcription

To determine if the downregulation of β-catenin expression affects its transcriptional activity, the expression of β-catenin target genes Cyclin D1 and c-myc were investigated since both have been shown to play a role in colon cancer. Neither Cyclin D1 nor c-myc mRNA (Table 2) or protein

Table 3. Changes in protein levels

	Protein (relative expression, ± SEM)		
	Control (n = 10)	SM (n = 20)	p
Bax	1233.2 ± 249.2	1627.6 ± 566.3	0.598
Bcl-2	**146.1 ± 57.0**	**544.7 ± 124.6**	**0.033**
Cyclin D1	5686.9 ± 369.5	5551.8 ± 368.1	0.809
c-myc	1230.0 ± 267.5	1296.4 ± 234.1	0.859
PKC-βI	3664.5 ± 604.7	2767.8 ± 716.4	0.394
PKC-βII	2466.5 ± 841.8	3040.1 ± 621.5	0.593

levels (Table 3) were affected by supplementation of the diet with SM. However, neither protein could be detected in the colon by immunohistochemistry, and Cyclin D1 was only detectable in the nuclei of both colonic adenomas and adenocarcinomas (data not shown).

3.4 Effects of sphingolipids on the expression of select genes involved in regulation of cell proliferation and death

To identify specific signaling pathways that are important for the reversal of carcinogen-induced changes in the rate of proliferation and apoptosis we have reported previously [13], the mRNA levels of select targets known to be regulated by sphingolipid metabolites *in vitro* were determined in the colonic mucosa. There were no statistically significant effects of SM supplements on the mRNA levels of the pro- (Bax, Bad), and anti-apoptotic genes (Bcl-2 and Bcl-XL) selected (Table 2). Since the generation of a pro-apoptotic state could involve changes in the ratio of pro- and anti-apoptotic genes rather than extensive changes in the expression of a single gene, the ratio of Bax plus Bad over Bcl-2 plus Bcl-XL was calculated. However, the difference in the mRNA ratio (1.39 ± 0.15 SEM in the SM-fed group *versus* 1.23 ± 0.17 SEM in the control group) was not statistically significant (p = 0.5487).

We then determined if orally administered SM can alter protein levels of Bax and Bcl-2 rather than their mRNA levels. As suggested by the mRNA levels, there was no statistically significant change in the bax protein levels (Table 3, Fig. 4). Surprisingly, the protein level of Bcl-2 was significantly elevated in the SM-fed mice (p = 0.033) (Table 3, Fig. 4), leading to a significantly lower ratio of Bax/Bcl-2 protein in the treated group (14.75 ± 3.82 and 4.73 ± 1.73 for control and SM-fed, respectively p = 0.023). A cleavage product of approximately 20 kDa with pro-apoptotic properties as described by Cheng *et al*. [25] was not detected.

In addition, putative upstream regulators of cell growth and death were analyzed. Here, PKC was chosen because its important role in many facets of cell regulation, and because it is a direct target of sphingolipids [26]; a role of the PKC-β isozymes in colon cancer has been suggested [27]. In the sphingolipid-fed groups, both PKC-βI (a splicing variant of PKC-βII) and PKC-βII mRNA levels were significantly increased (p = 0.025 and p = 0.036, respectively) (Table 2, bold). This, however, did not lead to a concurrent increase in either PKC-βI or PKC-βII protein levels in the colonic mucosa (Table 3).

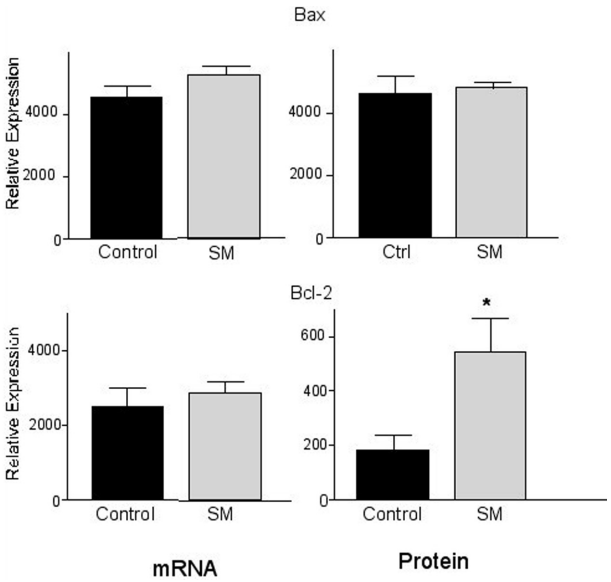

Figure 4. Upregulation of Bcl-2 protein levels in the colonic mucosa of sphingolipid-fed CF1 mice. Dietary SM did not affect mRNA levels of the anti-apoptotic protein Bcl-2 in the colonic mucosa of carcinogen-treated CF1 mice but increased its protein levels. *p = 0.033.

4 Discussion

Intestinal cells are constantly exposed to bioactive sphingolipid metabolites when dietary sphingolipids are hydrolyzed in all regions of the intestinal tract to ceramide and sphingosine [16, 27, 28] and regulate cell proliferation and death [10, 11]. These events were associated with the suppression of carcinogen-induced colon cancer in mice [11]. In the present study, expression levels in gene products that may mediate the sphingolipid-induced tumor suppression were evaluated to gain insight into the underlying mechanisms and signaling pathways induced by dietary sphingolipids in the prevention of early stages of colon cancer. Our data indicate that dietary SM did not play a role in the regulation of *in vivo* gene transcription in the colonic mucosa in most of the target genes we had analyzed. In contrast, we found that dietary SM selectively influenced the levels of specific proteins that are associated with the earliest stages of colon cancer. Rather than nonspecifically altering protein levels independent of the nature or function of the protein, SM specifically reversed β-catenin elevation and decreased connexin-43 protein and E-cadherin levels – changes that

are characteristic for early stages of colon carcinogenesis. The reversal of these early events may be critical for the preventive effect of orally administered sphingolipids on colon cancer.

β-Catenin accumulation caused by an *APC* or, more infrequently, a *CTNNB1* mutation is an early event in colon carcinogenesis as indicated by its cytoplasmic accumulation in macroscopically uninvolved colonic mucosa in Min mice [7, 14] or FAP patients [29]. β-catenin overexpression also appears to be essential for the progression of dysplastic ACF to adenomas [2] while its downregulation reverses the transformed properties of cells [30]. The effect of dysregulated β-catenin on progression may not only be related to its modulation of cell proliferation [31] but also to its function in cell adhesion and positioning of cells in the colonic crypts. An effect on signaling pathways regulating colonic functions and differentiation has also been suggested [32], emphasizing the importance of β-catenin during early stages of colon cancer. The downregulation of β-catenin by orally administered SM by post-transcriptional means demonstrated in the present study confirms our previously reported results in Min mice [14, 33]. The mechanisms of β-catenin downregulation are currently under investigation in our laboratory. Possible effects of sphingolipids are the increase in protein degradation or reduced stabilization. One target, the ubiquitin–proteasome system, has already been identified as a target for sphingolipids in yeast [34]; however, this was associated with the induction of apoptosis and therefore this pathway may not be relevant here. Another degradation pathway may be the activation of proteases, either directly or indirectly *via* PKC or other serine/threonine protein kinases and phosphatases that are targets of sphingolipids *in vitro*. Given the importance of b-catenin for the progression of the disease, the reversal of aberrant β-catenin expression may be one important mechanism to prevent colon cancer, and identifies β-catenin as a promising target for colon cancer prevention with sphingolipids.

β-Catenin that accumulates in the cytosol is translocated to the nucleus where it activates the transcription of genes that are involved in proliferation, adhesion and migration (see www.stanford.edu/~rnusse/wntwindow.html for a list of targets). In the present study, the fixation procedure of the colonic tissue did not allow for a determination of nuclear β-catenin in the colonic mucosa; instead, we investigated the effect of dietary SM on two targets of β-catenin-mediated transcriptional activity that have been implicated in colon cancer, cyclin D1, and c-myc. Although both c-myc and cyclin D1 can be downregulated by sphingolipid metabolites *in vitro* [35–37], dietary SM did not appear to influence their *in vivo* expression in the colonic mucosa. This suggests that the elevation of these proteins may not be among the critical early events associated with this model of colon carcinogenesis, and, thus, their downregulation may not be involved in the suppression of colon cancer by sphingolipids.

Connexins are a family of gap junctional proteins that form aqueous channels for the exchange of small hydrophilic molecules and ions between cells that enable cells to directly communicate and share signaling molecules. The impairment of gap junctional intercellular communication (GJIC) due to a lack of connexin-43 expression or loss of function is an early event in colon carcinogenesis [19, 24]. The restoration of GJIC has been shown to reverse phenotypical changes in cells and prevent tumor growth [38, 39], and therefore may be an important target in colon cancer prevention. Interestingly, connexin-43 is also a target of Wnt signaling and β-catenin [40]. In addition to the loss of connexin-43, loss of E-cadherin expression has also been associated with early stages of colon carcinogenesis. Our results indicate a trend toward increased E-cadherin protein levels in the sphingolipid-fed group. E-cadherin is instrumental in regulating intracellular trafficking of connexin-43 [41] and its loss affects connexin-43 expression and localization in cancer cells [42, 43]. Communication and adhesion proteins are especially important in colonic epithelial cells because of the necessity to connect to both underlying matrix and neighboring cells while moving along the crypt axis toward the lumen. Altering these fine-tuned events may cause the retention of transformed cells and support the formation of ACF and subsequently the formation of adenomas and adenocarcinomas.

Sphingolipids targets *in vitro* are serine/threonine protein kinases and phosphatases that regulate multiple major signaling pathways. Protein kinase C (PKC) was among the first identified targets of sphingolipids [26], and altered expression of PKC isozymes has been reported in various cancers. Of specific interest in this study was PKC-βII expression that is elevated both during tumor initiation and in colon carcinomas [44, 45] and contributes to increased proliferation of the colonic epithelium [2, 46]. Importantly, although RT-PCR analysis showed an increase in mRNA levels of both enzymes in the sphingolipid-fed mice, this did not correspond to increased protein levels. This confirms earlier results by Gokman-Polar [45]. Unfortunately, in this study, we could not determine whether sphingolipids alter the activity of the enzymes. However, this clearly warrants further investigation because PKC isozymes are also involved in the regulation of β-catenin metabolism [47].

The increase in Bcl-2 protein expression after feeding sphingolipids is in disagreement with many *in vitro* observations demonstrating that sphingolipid metabolites lower Bcl-2 and thereby facilitate apoptosis [48]. *In vivo*, both up- and downregulated Bcl-2 expression in colon tumors compared to the normal colonic mucosa have been reported [49, 50]. Furthermore, Bcl-2 expression has been associated with a better prognosis in colon [51]. Recent studies have demonstrated that an increase in Bcl-2 expression actually increases cell death by compromising the structural integrity of ER and mitochondria, and sensitized cells to apoptosis-inducing agents such as ceramide and staurosporin [52].

At this point it is not clear if this effect of Bcl-2 in both the *in vitro* and *in vivo* studies is the result of the cleavage of the protein by Caspase-3 to a bax-like, pro-apoptotic product [25] or the inactivation *via* dephosphorylation (perhaps of serine 70) by PP2A [53]. The effect of sphingolipid concentrations that do not induce apoptosis – as used in the present study – on both protein size and phosphorylation status will be determined in more detail to uncover its relevance in sphingolipid-mediated colon cancer prevention.

In summary, dietary SM specifically prevents or reverses aberrant expression of proteins associated with early stages of colon cancer by modulating post-transcriptional and/or post-translational events. These changes may be critical for the chemopreventive effect of orally administered sphingolipids, and their use of biomarkers for *in vivo* efficacy of orally administered sphingolipids are currently under investigation.

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