Fatty Acid CoA Ligase 4 Is Up-Regulated in Colon Adenocarcinoma

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ABSTRACT

Arachidonic acid metabolism plays an important role in colon carcinogenesis. Cyclooxygenase-2 (COX-2), which catalyzes the rate-limiting step in the synthesis of prostaglandins from arachidonic acids, is known to be up-regulated in colon cancer, and multiple lines of evidence indicate that it is a critical early step in colon carcinogenesis. Recently, 15-lipoxygenase-1, the enzyme that converts arachidonic acid to 15(S)-HETE, was also found to be up-regulated in colon carcinoma. In our previous studies, we cloned a gene that encodes another arachidonic acid-using enzyme, fatty acid CoA ligase 4 (FACL4), and showed that overexpression of this enzyme prevents apoptosis. We have also showed that FACL4 and COX-2 synergistically inhibit apoptosis by reducing the intracellular level of free arachidonic acid. Here, we report that expression of FACL4 is significantly increased in colon adenocarcinoma compared with adjacent normal tissue at both the mRNA and protein levels by quantitative RT-PCR (paired t test, P < 0.015), immunoblot, and immunohistochemical staining. We found that the increase in expression level of FACL4 mRNA relative to control ranged between 2.4- and 54.5-fold; the average fold-increase was 13.4. The increase in FACL4 protein expression is between 2.4- and 65.0-fold. In addition, we found that a higher level of increased FACL4 expression was correlated with well and moderately differentiated adenocarcinoma, whereas no similar correlation was observed with COX-2 expression. The in situ hybridization results indicate that expression of FACL4 is localized predominantly in the colon epithelium but not in the stroma. The onset of FACL4 up-regulation appears to occur during the transformation from adenoma to adenocarcinoma because FACL4 expression was not increased above normal in the three colon adenomas examined. Finally, we observed that a tumor promoter significantly induced FACL4 expression. These findings suggest that the FACL4 pathway may be important in colon carcinogenesis, and that the development of selective inhibitors for FACL4 may be a worthy effort in the prevention and treatment of colon cancer.

INTRODUCTION

Colorectal cancer is the third leading cause of cancer incidence and death in the United States, where 130,000 new cases are diagnosed each year, and half of the patients will die of the disease (1). NSAIDs 3 are effective in reduction of the incidence and mortality as shown in both epidemiological and genetic studies. Long-term intake of NSAIDs greatly reduced the risk of colon cancer incidence in the human population (2, 3), and administration of NSAIDs to patients with familial adenomatous polyposis led to remarkable regression of polyps (4, 5). In addition, NSAIDs also significantly reduced the size and number of polyps in chemical carcinogen-treated animals (6, 7). One major mechanism for the action of NSAIDs is inhibition of COX-2 activity. COX-2 is the inducible isoform of cyclooxygenase that catalyzes the rate-limiting step in the conversion of AA to prostanooids. Several lines of evidence indicate that the COX-2 pathway is important in colon carcinogenesis. COX-2 has been found to be up-regulated in colorectal and several other types of cancers (8–12). Moreover, a COX-2 null mutation caused a significant reduction in the size and number of polyps in APC mutant mice (13), and treatment with COX-2-specific inhibitors caused a dose-dependent reduction in the number and size of intestinal and colonic polyps in APC mutant mice (14). These studies in animal models strongly suggest that induction of COX-2 is an early step in colon carcinogenesis.

In addition to the COX-2 pathway, other metabolic pathways that use AA as a substrate also have been shown to be associated with carcinogenesis. For example, the reticulocyte type 15-LOX was found to be expressed at high levels in colorectal cancer epithelial cells (15). Blocking the LOX pathways with antisense oligonucleotides or with LOX inhibitors has been shown to induce apoptosis in rat W256 carcinosarcoma cells (16). Similarly, inhibition of the 5-LOX pathway by MK886 induced massive apoptosis in prostate cancer cell lines (17). In recent studies, we found that NSAIDs and triacsin C, an inhibitor of another AA-using enzyme, long-chain FACL, have a synergistic effect in induction of apoptosis in the colon cancer cell line HT29 (18). FACL converts fatty acids to fatty acyl-CoA esters, which function mainly as activated lipid intermediates for the formation of complex lipids and as precursors for fatty acid β-oxidation. An early study with the inhibitor triacsin C indicated that inhibition of the FACL pathway led to a reduction in lipid synthesis and to inhibition of cell proliferation (19). In vitro and in vivo studies suggest that long-chain fatty acyl-CoA esters can also act as regulatory molecules in signal transduction and transcriptional activation and modulate a number of cellular functions including membrane fusion and ion transportation (20, 21). We previously cloned the gene encoding the FACL isoform that highly prefers AA among other fatty acids as substrate, FACL4 (22). The additive effect of the inhibitors of COX and FACL prompted us to hypothesize that intracellular free AA signals apoptosis and that metabolically lowering the level of intracellular free AA prevents apoptosis. In an inducible cell expression system, we showed that overexpression of COX-2 and/or FACL4 prevented apoptosis by reducing the level of the intracellular free AA (18). Because COX-2 has been found to be up-regulated in colon carcinoma, an important question is whether FACL4 is also up-regulated in colon carcinoma. In this study, we examined the expression level and localization of FACL4 in colon adenocarcinoma and compared it with COX-2. We found that expression of FACL4 is significantly increased in colon adenocarcinoma compared with the adjacent normal tissue. The majority of FACL4 expression is found in the colon epithelium but not in the stroma. Moreover, no increase in FACL4 expression was observed in all adenomas examined, suggesting that FACL4 could be a potential molecular marker for the early onset of colon adenocarcinoma.

MATERIALS AND METHODS

Colon Adenocarcinoma Samples. Frozen colon adenocarcinoma samples were obtained from the Tissue Procurement Core Facility at the Huntsman Cancer Institute. Each tumor and the adjacent normal tissue were removed surgically from the patient, snap-frozen, and stored at −70°C. A complete histological examination was performed on the tissue sections, and surplus portions were used for the experiments reported here.

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2 To whom requests for reprints should be addressed, at Huntsman Cancer Institute, 2000 Circle of Hope, Room 5344, University of Utah, Salt Lake City, UT 84112. Phone: (801) 585-0331; Fax: (801) 585-6345; E-mail: ccao@hci.utah.edu.

3 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; FACL4, fatty acid CoA ligase 4; COX-2, cyclooxygenase 2; RT-PCR, reverse transcription-PCR; AA, arachidonic acid; PMA, phorbol 12-myristate 13-acetate; LOX, lipoxygenase; RT-PCR, reverse transcription-PCR.
Preparation of RNA from Colon Tissues. Frozen colon tissues were thawed and mixed with TRIzol Reagent (Life Technologies, Inc.) and then homogenized in a Dounce homogenizer for 50 strokes on ice. The homogenate was transferred to a 1.5-ml tube and centrifuged for 10 min at 14,000 rpm. The supernatant was transferred to a new tube, and isolation of RNA was performed following the manufacturer’s instructions for TRIzol Reagent.

Preparation of Protein Lysate from Colon Tissues. Frozen tissue was thawed, and lysate was prepared by sonication as described (23). The protein concentration of the supernatant was measured using the BCA protein assay (Pierce). Immuno blot analysis for FACL4 was performed as described previously (24).

Quantitative RT-PCR. Quantitative RT-PCR was performed with a QuantumRNA β-actin internal control (Ambion, Inc.). The ratio of primer/competi- tive for β-actin was 3:7. RT-PCR was carried out using primes correspond- ing to the coding sequences of human FACL4 or COX-2. The sizes of the amplified fragments for FACL4, COX-2, and FACL1 were 441, 480, and 511 bp, respectively; the β-actin primers amplify a fragment of 294 bp. The primers used in this study were: FACL4, sense (AAG GAA GCA AAG GAG ACT GTA) and antisense (TCT TGC CAG TCT ATT AGC TTA); COX-2, sense (TTG TTG AAT CAT TCA CCA GGC) and antisense (ACA CTG AAT GAA GTA AAG GGA); and FACL1, sense (AGC CCT TGG TGT ATT TCT ATG) and antisense (CCG CTT CAT GCT GGT GAC TTC). In Situ Hybridization. The procedure was performed as described previ- ously (24). To prepare the RNA probes for hybridization, the construct pSL79, which contains human FACL4 NH2-terminal sequence, was digested with EcoRV, and the 3.5-kb fragment was purified. An antisense RNA probe of 682 bp (corresponding to nucleotides +866 to +1548) was generated by in vitro transcription catalyzed by T3 RNA polymerase. To generate a sense probe of 1024 bp (corresponding to nucleotides −317 to +707), the same plasmid was digested with SphI, the 3.9-kb fragment was purified, and in vitro transcription was carried out with T7 RNA polymerase. For RNA probes of COX-2, a cDNA construct containing the 5′ untranslated region and NH2-terminal sequence of human COX-2 (HincII/HincII fragment corresponding to nucleotides −108 to +305) was linearized by HindIII, and an antisense RNA probe of ~450 bp was generated by in vitro transcription catalyzed by T3 RNA polymerase. A complementary sense RNA probe was generated for T7 RNA polymerase- catalyzed in vitro transcription after linearization of the plasmid with XhoI.

Fluorescent Immunohistochemical Staining. Fresh colon crypts were isolated as described previously (25). Isolated crypts were fixed in 2% paraformaldehyde for 1 h, washed with PBS buffer, and stored at 4°C. They were then transferred to tissue adhesive printed slides (Electron Microscopy Sciences) and permeabilized with 0.2% Triton X-100 (in PBS) at room temperature for 1 h. Slides were washed three times for 1 h at room temperature and blocked with 5% goat serum overnight at 4°C. The anti-FACL4 antibody (24) was diluted 1:200, applied to the slides, and incubated overnight. An FITC-conjugated antibact antibody (Vector Laboratories, Inc.) was diluted 1:200 and incubated in dark at room temperature for 1.5 h. The slides were washed with PBS, and Texas Red-X phallidin (Molecular Probes, 1:100) was added. Negative controls consisted of omitting the anti-FACL4 antibody, and/or secondary antibody, or preincubating the antibody with the immunizing peptide overnight. Slides were mounted with the Prolong anti-fade kit (Molecular Probes) and air-dried. Staining was viewed by a Fluoview scanning laser microscope (Olympus).

Statistical Analysis. The data were analyzed using SAS programming software (SAS Institute, Inc., Cary, NC). The subjects were first treated as the effects of binary variables by univariate methods, and paired t test and matched-pairs Wilcoxon test were performed. The data were then subjected to multivariate analysis using a general linear mixed model. Measurements on the same subjects were taken to determine a possible subject-specific effect.

RESULTS

FACL4 Is Up-Regulated in Colon Adenocarcinoma. To quantify the mRNA level of FACL4 in colon tissues, we adopted a quantitative RT-PCR system that allows us to carry out multiplex RT-PCR with an endogenous standard (β-actin) for internal normalization. The advantage of this system is that the amplification efficiency of the β-actin primer can be modulated by including the competimers, and this will overcome the high abundance of β-actin transcripts and make it feasible to amplify with target transcripts of less abundance. For each sample, multiple PCRs were performed, such that the intensity of the observed amplified product was linear with respect to cycle number. Fig. 1A is a representative RT-PCR quantification in which the expression level of FACL4 mRNA in three pairs of colon tissue samples was examined. Each colon adenocarcinoma was compared with the normal tissue surrounding the tumor in the same patient. The identity of the amplified fragment was verified as a partial sequence of the human FACL4 gene by sequencing analysis (not shown). RT-PCR results from 26 pairs of samples were quantified by densitometry analysis, and the results are shown in Table 1. To analyze the expression level of FACL4 protein, we prepared total protein lysate from the colon adenocarcinoma and the paired normal control tissues and performed immunoblotting with the anti-FACL4 antibody as described previously (24). A representative immunoblot with three pairs of colon samples is shown in Fig. 1B, and the densitometry analysis of all of the immunoblot results is summarized in Table 1. Quantification of the RT-PCR results indicated that 25 of 26 paired samples had at least a 2-fold higher expression level in the adenocarcinomas. FACL4 expression is significantly higher in colon adenocarcinomas compared with the normal tissues (paired t test, P < 0.015). The change in the expression level of FACL4 relative to control ranged between 2.4- and 54.5-fold; the average fold increase was 13.4. Furthermore, immunoblot results revealed that FACL4 protein expression was significantly higher in adenocarcinomas, with a fold increase ranging between 2.4 and 65.0 relative to control. In the 22 paired samples we examined by immunoblotting, only two adenocarcinomas (cases 243 and 204) showed an increase in FACL4 mRNA expression but no change in FACL4 protein expres- sion. By paired t test and matched-pairs Wilcoxon test, we found that there was no correlation between the expression level of FACL4 and either the sex (P > 0.19) or age (P > 0.15) of the patient. Statistical analysis revealed that the fold increase in FACL4 expression corre- lated with the state of differentiation of the adenocarcinomas (P = 0.07), in contrast, no such correlation was evident for COX-2 (P > 0.40). Moreover, we determined the level of COX-2 mRNA expression in some of the adenocarcinoma samples and found that no significant correlation existed between the fold increase of FACL4 and COX-2 mRNA expression (Spearman’s correlation coefficient 0.20, P = 0.70).

![Fig. 1. FACL4 expression is elevated in colon adenocarcinoma. A, a representative quantitative RT-PCR analysis. Total RNA was extracted from colon adenocarcinoma and paired adjacent normal tissues. cDNA was then synthesized and amplified with the FACL4 amplicon for 32 and 35 cycles, as indicated. β-actin was amplified as an internal normalization standard. B, a representative immunoblot. Total protein lysate was isolated from adenocarcinoma and paired normal tissues from the same patient (see “Materials and Methods”), separated by SDS-PAGE, and blotted with an anti-FACL4 antibody and an anti-β-actin antibody for normalization (24). C, colon adenocarcinoma; N, normal colon tissue from the same patient. Ctrl, a control cell lysate indicating the position of the FACL4 band. The number on top of each panel corresponds to the case number in Table 1. Densitometry analysis of the RT-PCR and the immunoblot results of 26 pairs of carcino- nomas is shown in Table 1.](https://cancerres.aacrjournals.org/article/000/000/000/000/000/000)
To determine where and in what type of cells in colon adenocarcinomas FACL4 is expressed, we performed *in situ* hybridization and immunohistochemical staining. The *in situ* hybridization results revealed that FACL4 is expressed in both normal and adenocarcinoma tissues, but that the expression level is much higher in the adenocarcinomas (Fig. 2, A and B). Control hybridizations with sense probe indicated that the FACL4 signal is specific (Fig. 2 C). The location of FACL4 is predominantly in the colon epithelium; the signal in the stromal fibroblast and proinflammatory cells is very weak (Fig. 2 A).

We observed a similar expression pattern of FACL4 protein by immunostaining with an anti-FACL4 antibody (data not shown). The distribution of COX-2 mRNA was also examined by *in situ* hybridization and compared with the distribution pattern of FACL4 mRNA in serial sections of colon adenocarcinoma tissues (Fig. 2, D–F). The similar expression pattern of both enzymes suggests that FACL4 may be colocalized with COX-2. To control for the potential problem of protein degradation in frozen tissue sections, we also carried out immunohistochemical staining with freshly isolated colon crypts (Fig. 3). To reveal the colon crypt structure, we used phaloidin to indicate the location of actin.

### Table 1: Quantification of FACL4 and COX-2 expression in colon adenoma and adenocarcinoma patients

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Tumor characteristics</th>
<th>Histology</th>
<th>Differentiation</th>
<th>RT-PCR</th>
<th>Western blot</th>
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<tr>
<td>148</td>
<td>70</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>Poor</td>
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<td>0.298</td>
<td>0.515</td>
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<tr>
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<td>N: 0.025</td>
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<td>1.124</td>
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<td>N: 0.077</td>
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<td>0.145</td>
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<tr>
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<td>0.537</td>
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<td>0.084</td>
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<tr>
<td>119</td>
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<td>M</td>
<td>Tubular adenoma to adenocarcinoma</td>
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<td>0.113</td>
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<td>482</td>
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<td>Adenoma</td>
<td></td>
<td>A: 0.519</td>
<td>0.016</td>
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* Paired normal colon tissue.
* The PCR amplified bands were quantified using the Kodak Science software. The number shown is calculated as a ratio between the intensity of the amplified target gene and the β-actin gene.
* Colon adenocarcinoma.
* Information is not available.
* Colon adenoma.
normal colon crypt was weak. In contrast, staining in the disrupted colon crypts from adenocarcinoma tissues was more intensive and exhibited a granular pattern, which was also sometimes observed in the normal colon epithelium (Fig. 3). The staining is specific with the anti-FACL4 antibody because negative staining was observed when the antibody was preincubated with a peptide corresponding to the NH$_2$-terminal FACL4 sequence.

**Expression of FACL4 Did Not Increase in Colon Adenoma.** We obtained 2 pairs of colon adenoma tissues (cases 363 and 482; Table 1) with no histological characteristics of malignant transformation and 1 pair (case 119) that showed a tubulovillous adenoma with a small focal area of moderately differentiated adenocarcinoma in multiple sections from the tumor mass. Quantitative RT-PCR analysis showed that FACL4 mRNA was not increased in the adenomas compared with the adjacent normal tissue (Fig. 4A; Table 1). Immunoblot results with the anti-FACL4 antibody also revealed that the FACL4 protein level was not elevated in adenomas (cases 363 and 482). In case 119, a slight increase in FACL4 protein expression was detected (Fig. 4B; Table 1). This is presumably because this tumor sample is at an intermediate stage between adenoma and adenocarcinoma. We also determined the expression of COX-2 in these three paired samples and found that COX-2 expression was up-regulated in 2 cases (Fig. 4A; Table 1). This suggests that the onset of COX-2 up-regulation is earlier than that of FACL4 in colon cancer development.

**Comparison of FACL4 and FACL1 Expression in Colon Adenocarcinoma.** To examine whether the elevated expression of FACL in colon adenocarcinoma is limited to isoform 4, we quantified FACL1 mRNA expression by RT-PCR analysis. Unlike FACL4,
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To examine whether elevated expression of FACL4 in cancerous epithelial cells can be achieved in vitro, we treated Int407 cells, an intestinal epithelial cell line, with the tumor promoter PMA. Quantitative RT-PCR analysis showed that FACL4 was induced as early as 2 h after PMA treatment (Fig. 6A). We also detected a significant increase in FACL4 protein after 24-h treatment by PMA (Fig. 6B). The time dependence and dose response of FACL4 induction by PMA is similar to that of COX-2 as shown in Fig. 6A, suggesting that FACL4 is an immediate early response gene to tumor promoter.

**DISCUSSION**

In this study we found that FACL4, an AA-using enzyme, is significantly up-regulated in colon adenocarcinomas. To our knowledge, this is the first report that FACL4 expression is highly elevated in cancer. We also examined the expression of another isoform of FACL, FACL1, and found that up-regulation of this isoform does not significantly correlate with adenocarcinoma, suggesting that not every isoform from this enzyme family is up-regulated in colon adenocarcinomas. The predominant location of FACL4 is in colon epithelium, which is similar to the localization of COX-2. Moreover, the up-regulation of FACL4 observed in cancerous epithelium can be reproduced in an intestinal epithelial cell line exposed to the tumor promoter PMA.

In three pairs of adenoma samples examined, no increase in FACL4 expression was detected; thus, the onset for the abnormal regulation of FACL4 appears to be at a stage between colon adenoma and adenocarcinoma. This suggests that elevated expression of FACL4 is potentially a clinically useful molecular predictor of colon cancer progression. The expression of COX-2 was elevated in 2 of the 3 adenoma samples, indicating that induction of COX-2 is an earlier molecular event than the enhanced expression of FACL4 in colon cancer progression, although more adenoma samples are needed to confirm this conclusion. We also examined FACL4 expression in other epithelial cancers, including breast and kidney carcinomas. Statistical analysis of the results from RT-PCR quantification indicates that FACL4 expression was not increased in the breast or kidney cancer cells compared with the normal surrounding tissue from the same patient (not shown). This suggests that among epithelial cancers, FACL4 is probably specifically up-regulated in colon cancer. However, the up-regulation of FACL4 is probably not limited to colon cancer because we also found that FACL4 is highly expressed in squamous and basal cell carcinomas of the skin relative to control (not shown).

What is the mechanism for the elevated expression of FACL4 in colon cancer? We observed that both FACL4 mRNA and protein were
highly expressed in colon adenocarcinoma compared with the adjacent normal tissue, indicating that the mechanism for this regulation is predominantly at a step before translation. We speculate that the mechanism for up-regulation of FACL4 is via activation of oncogenes or inactivation of tumor suppressors. Tumor suppressor APC inactivating mutations are found in a great majority of familial adenomatous polyposis and sporadic colorectal cancers (27, 28). Inactivation of this gene is one of the earliest events in colon carcinogenesis (29). K-ras oncogene is activated during the progression of colon adenoma to adenocarcinoma, which is believed to be at a stage later than APC mutation (29), and moreover, COX-2 can be induced in Ras-transformed epithelial cells (30). Therefore, our speculation is that components in the K-ras and/or APC pathways are the potential regulators for FACL4 up-regulation in colon carcinoma.

Does elevated FACL4 expression play any causal role in colon carcinogenesis? We have observed a synergistic effect of FACL4 and COX-2 inhibitors in the induction of apoptosis, and we have also demonstrated that overexpression of either FACL4 or COX-2 prevents apoptosis (18). More importantly, simultaneous activation of both FACL4 and COX-2 pathways had a synergistic effect in inhibition of apoptosis attributable to the removal of intracellular free AA. Because attenuation of apoptosis is an important mechanism for colon epithelial transformation, coordinated up-regulation of COX-2, FACL4, and perhaps also other AA-utilizing enzymes might promote colon cancer development by inhibition of apoptosis. In addition to removing intracellular free AA, it is evident that the COX product prostaglandin E2 contributes to the preventive action of COX-2 in apoptosis. In human colon cancer cells, prostaglandin E2 inhibited apoptosis (31). The FACL4 pathway generates fatty acyl-CoA esters, and the most prevalent products of this enzyme isoform are arachidonoyl-CoA esters. These fatty acyl-CoA esters have been shown to modulate transcription, to regulate key signaling molecules such as protein kinase C (20), and to directly bind to transcription factors (32).

The multiple molecular actions of the fatty acyl-CoA esters raise the possibility that similar to the COX-2 product prostaglandin E2, the FACL4 products might also play roles in promoting cell proliferation and cell growth. Our report suggests a potentially important role for FACL4 in colon carcinogenesis. Therefore, development of selective FACL4 inhibitors and evaluation of its use alone or in combination with other chemotherapeutic drugs could lead to a novel approach for treatment and prevention of colon cancer.

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