Carcinogenesis vol.20 no.10 pp.1985–1995, 1999

Decreased 13-*S*-hydroxyoctadecadienoic acid levels and 15-lipoxygenase-1 expression in human colon cancers

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13-*S*-Hydroxyoctadecadienoic acid (13-S-HODE), the product of 15-lipoxygenase (15-LOX) metabolism of linoleic acid, enhances cellular mitogenic responses to certain growth factors. Other observations have questioned whether 13-S-HODE has tumorigenic effects. Our study evaluated the hypothesis that 15-LOX-1 is overexpressed in colon cancers resulting in an increase in intracellular 13-S-HODE. 15-LOX-1 and 13-S-HODE were quantified using western blots, ELISA and immunohistochemistry in 18 human colon cancers with paired normal colonic mucosa. Additionally, 15-LOX-1 expression was measured by western blots in three transformed colonic cell lines and in a human umbilical vein endothelial cell line. Next, we evaluated 13-S-HODE effects on cellular proliferation, cell cycle distribution and apoptosis in a transformed colonic cell line (RKO). Cell cycle distributions were measured by flow cytometry and apoptosis was assessed by phase contrast microscopy, electron microscopy, flow cytometry and DNA fragmentation assay. 15-LOX-1 immunohistochemistry staining scores were reduced in tumor tissues ($P \leq 0.0001$) and 15-LOX-1 expression was absent in three transformed colonic cell lines. 13-S-HODE levels were also reduced in tumors tissues compared with normal controls by ELISA (median 3.3-fold, $P = 0.02$) and by immunohistochemistry ($P \leq 0.0001$). *In vitro* 13-S-HODE inhibited RKO cell proliferation and induced cell cycle arrest and apoptosis. 13-S-HODE produced similar effects in HT-29 cells. Our observations indicate that: (i) human colon cancers are associated with a down-regulation in 15-LOX-1 expression and a reduction in 13-S-HODE intracellular levels; (ii) 13-S-HODE can suppress cell proliferation and induce apoptosis in transformed colonic epithelial cells.

Abbreviations: DAB, 3,3′-diaminobenzidine; EGF, epidermal growth factor; H&E, hematoxylin and eosin; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; 12-S-HETE, 12-*S*-hydroxyeicosatetraenoic acid; 13-S-HODE, 13-*S*-hydroxyoctadecadienoic acid; 15-LOX, 15-lipoxygenase; NDGA, nordihydroguaiaretic acid; PBS, phosphate-buffered saline; NaBT, sodium butyrate; SHE, Syrian hamster embryo; TBS, Tris-buffered saline.

Disclosure statement: Dr Ramesh G.Reddy and Mr Stephen A.Spindler are previous employees of Oxford Biomedical Research Inc., which manufactures the 13-S-HODE kits.

Introduction

A diet high in fats increases the risk of colorectal cancer, as shown in several epidemiological studies (1). Among dietary fats, polyunsaturated fatty acids in particular enhance carcinogen induction of intestinal tumors (2,3). The major polyunsaturated fatty acid in the human diet is linoleic acid (4). Linoleic acid promotes carcinogenesis in azoxymethane rat models and the growth of murine adenocarcinomas in nude mice (5,6). Oxidation of polyunsaturated fatty acids is necessary for them to stimulate mitogenesis in rat rectal epithelia (7,8). Linoleic acid oxidation is predominantly mediated by 15-lipoxygenase (15-LOX) and produces 13-*S*-hydroxyoctadecadienoic acid (13-S-HODE) in humans (9,10). Recently, a second lipoxygenase has been described and classified as 15-LOX-2 (11), while the formerly known reticulocyte 15-LOX has been renamed 15-LOX-1. Human colon tissues have been found to express 15-LOX-1 but not 15-LOX-2 (11,12).

Several lines of evidence suggest a link between lipoxygenase metabolism of linoleic acid and tumor promotion by linoleic acid. First, lipoxygenase inhibitors attenuate linoleic acid stimulation of murine adenocarcinoma growth (13). Second, 15-LOX formation of 13-S-HODE enhances the mitogenic response to epidermal growth factor (EGF) in BALB/c3T3 fibroblasts (14) and Syrian hamster embryo (SHE) cells (15). Similarly, EGF and transforming growth factor α stimulate DNA synthesis and 13-S-HODE production in transformed breast cancer cells (BT-20) (16). Third, 13-S-HODE production increases when normal fibroblasts are transfected with c-erbB-2 (a proto-oncogene similar to EGF) (17). Finally, studies of 13-HODE dehydrogenase, the enzyme that converts 13-S-HODE to 13-oxoctadienoic acid, have also suggested an association between 13-S-HODE metabolism and colon carcinogenesis. Colonic tissues are one of two sites that have the highest level of 13-HODE dehydrogenase activity in rats (4) and 13-HODE dehydrogenase activity levels correlate positively with the degree of colonic cell differentiation (18). Correspondingly, human colonic cancer tissues have lower levels of 13-HODE dehydrogenase activity compared with normal tissues (19). Based on these observations, it has been proposed that 13-S-HODE promotes colonic cell proliferation, while 13-HODE dehydrogenase inactivates 13-S-HODE to allow colonic cellular differentiation (19).

Despite these observations which imply that linoleic acid might enhance tumorigenesis through 13-S-HODE, a direct link between 13-S-HODE and colonic carcinogenesis has not been demonstrated. Additionally, some observations have questioned whether 13-S-HODE has tumorigenic effects (20), including studies on transformed SHE cells which had lost a

tumor suppresser gene function. In these cells 13-S-HODE failed to augment EGF-dependent DNA synthesis as in the normal phenotype SHE cells (21). Furthermore, the epithelial levels of 13-S-HODE in cancerous and normal human colon tissues are still unknown. Therefore, this study has examined the hypothesis that 15-LOX-1 is up-regulated in the transformed colonic epithelium, which leads to an increase in intracellular levels of 13-S-HODE. We expected 15-LOX-1 expression and 13-S-HODE levels to be higher in colonic cancer tissues than in their normal counterparts.

Materials and methods

Sample acquisition

Following institutional review board approval, samples were obtained through the University of Michigan Cancer Center tissue procurement program from surgical tissue specimens of 18 subjects who underwent colonic resection for colon cancers at the University of Michigan Medical Center. In each case, samples were procured from both the tumor area and from normal-appearing mucosa. The distance between the sampled normal-appearing mucosa and the center of the tumor was >10 cm for 16 of 18 subjects (except for cases 13 and 17). Tissue blocks were frozen at –70°C until the time of processing. Frozen sections were cut at –20°C. A staff pathologist at the University of Michigan Medical Center (J.G.) confirmed the locations of normal and cancerous epithelia in each sample on hematoxylin and eosin (H&E) stained sections. Samples for ELISA and western blotting were dissected at –20°C from normal and tumorous epithelia, which were localized on each tissue block by H&E staining.

Materials

Rabbit polyclonal antiserum to human recombinant 15-LOX-1 and standards of recombinant 15-LOX-1 were a generous gift from Dr Elliot Sigal and Dr Mary Mulkins (Roche Bioscience, Palo Alto, CA) (22). The specificity of the antibody against recombinant human reticulocyte 15-LOX-1 has been well characterized and the antibody has been used extensively in published studies on 15-LOX-1 in humans (22–26). Sheep polyclonal antibody against rabbit reticulocyte 15-LOX-1 and standard 13-S-HODE solution were obtained from Cayman Chemical (Ann Arbor, MI). 10% SDS–PAGE pre-cast gels were purchased from Bio-Rad (Richmond, CA). Antiprotease cocktail tablets were obtained from Boehringer Mannheim (Indianapolis, IN). Vectastain ABC kits and secondary anti-rabbit and anti-goat antibodies were purchased from Vector Laboratories (Burlingame, CA). 13-S-HODE antibody raised in goat and 13-S-HODE kits were supplied by Oxford Biomedical Research (Oxford, MI). The specificity of the 13-S-HODE antibody has been well demonstrated in prior studies (27). Transformed colonic cell lines HT-29 and SW-620 were obtained from American Type Culture Collection (Rockville, MD). The RKO rectal carcinoma cell line was kindly provided by Dr Albert J. Fornace (NCI, Bethesda, MD). The human umbilical vein endothelial cell (HUVEC) line was purchased from Clonetics (San Diego, CA). Other reagents, molecular grade solvents and chemicals were obtained from regular commercial manufacturers.

Determination of 13-S-HODE levels by ELISA

13-S-HODE was extracted from each tissue sample at 4°C as follows. Each frozen tissue specimen was placed in 250 µl phosphate-buffered saline (PBS), homogenized at low speed for 1 min and centrifuged at 2100 *g* for 30 s. Protein concentration was determined by the method of Bradford (28). The solution was acidified to a pH of 3.5–4.0 with 0.2 M HCl solution. The organic phase of the solution was extracted using water-saturated ethyl acetate. Samples were dried completely in a centrifuge dryer, then reconstituted with a mixture of 25 µl methanol, 975 µl dilution buffer (provided with the 13-S-HODE kits) and 50 µl chloroform. The pH was adjusted to 7.2 and the samples were stored at –20°C. 13-S-HODE commercial plates pre-coated with anti-13-HODE antibodies were used to measure 13-S-HODE levels in tissue extracts at room temperature. Serial dilutions of 13-S-HODE tissue extracts were prepared and 100 µl volumes of each dilution were added to one of five wells. An aliquot of 100 µl of a 13-S-HODE–horseradish peroxidase (HRP) conjugate (1:1000) was added to each well and the plates were incubated for 2 h at room temperature. Wells were washed twice with wash buffer, then 200 µl of 3,3′,5,5′-tetramethylbenzidine reagent was added. The plates were then incubated for 20 min at room temperature. The reaction was terminated by adding 50 µl of 1 N sulfuric acid. The absorbance was measured using a microtiter plate reader at 450 nm. Concentrations of 13-S-HODE were normalized per µg of protein of the tissue sample.

Western blot of 15-LOX-1 protein

The method was modified from Lei and Rao (24). The tissue samples were processed at 4°C. Each sample was placed in 500 µl of antiprotease cocktail solution, homogenized at low speed for ~1 min, sonicated for 10 s three times and then centrifuged at 10 000 *g* for 15 min. Total protein concentration was determined using the method of Bradford (28). An aliquot of 100 µg of crude protein from each specimen was immunoprecipitated using 15-LOX-1 antibody against rabbit reticulocyte 15-LOX-1 (Cayman Chemical). Equal amounts (50 µg of crude protein) of each immunoprecipitate were subjected to electrophoresis on a SDS–polyacrylamide gel (10%) under reducing conditions using a Bio-Rad Miniprotein Gel Electrophoresis Unit. Tumor and normal samples from the same subject were separated on the same gel. To verify the identification of 15-LOX-1 proteins, recombinant human 15-LOX-1 standard (50 ng) and molecular markers were run on each gel, while one or two lanes were loaded with sample buffer solution only, as a negative control. The separated proteins were electroblotted onto nitrocellulose membrane using a Bio-Rad Mini-Transfer Unit. The blots were incubated with 20% (w/v) non-fat dry milk in PBS overnight. After three washes with Tris-buffered saline (TBS) containing 0.05% Tween 20, the membranes were incubated in a 1:2000 dilution of 15-LOX-1 antibody (Roche Bioscience) at room temperature for 3 h with slow agitation. The blots were washed with TBS/Tween (0.05%) three times and re-incubated for 1 h at room temperature with anti-rabbit IgG–HRP conjugate. Blots were washed in TBS/Tween (0.05%) three times and the films were developed by the Enhanced Chemiluminescence (Amersham, Arlington Heights, IL) method. Resulting bands were quantified using NIH Image (NIH Freeware). The blots were digitized using a scanner linked to a Macintosh computer. The digitized blots were then uploaded into Image and each sample autograph was quantified by using the Integrated Density function. This function automatically subtracts background density from the blot density and provides a quantified gray scale for each pixel in the outlined band. The ratio of areas under the curve of the unknown to the standard on each membrane was used to estimate the amount of the unknown. Separate experiments showed a linear relationship between known amounts of 15-LOX-1 and areas under the curve within the measured concentration range.

Preparation of cell line samples for western blotting

HUVEC were grown to confluence using medium and growth factors from Clonetics on fibronectin-coated flasks. Cells were dislodged and lysed in a solution of 150 mM NaCl, 50 mM Tris–HCl, 0.5% Triton X-100 (pH 7.4) and antiprotease. Transformed cell lines (RKO, HT-29 and SW-620) were grown in 100 mm tissue culture dishes to confluence. The cells were lysed using an NP-40 buffer solution containing protease inhibitors. Protein was measured in all cell lines by the Bradford method (28). Western blotting was performed as described for tissue samples.

15-LOX-1 protein expression by immunohistochemistry

Frozen sections of 5 µm thickness were cut at –20°C, air dried and fixed in acetone for 30 s. At the time of staining the sections were incubated with 3% H_2O_2 in ethanol for 30 min to inactivate endogenous peroxides. Non-specific antibody binding sites were blocked using 20% goat serum in PBS for 30 min. Tissue sections were incubated in (1:8000) 15-LOX-1 primary antibody solution in a humidified chamber at 4°C. The next morning they were washed with PBS and then incubated for 30 min with the secondary antibody solution (1:100 biotinylated anti-rabbit antibody; Vector Laboratories). An avidin–biotin–immunoperoxidase complex solution (ABC complex; Vector Laboratories) was applied for 15 min at room temperature, washed with PBS and slides were re-incubated in a solution of 0.1 M 3,3′-diaminobenzidine (DAB) in 0.05 M TBS with 0.5 ml 3% H_2O_2 DAB solution (Vector Laboratories) for 5 min. Slides were counterstained with hematoxylin. The primary antibody solution was substituted with PBS or rabbit serum in negative control experiments. Frozen lung sections were stained for positive control experiments. Staining was evaluated and rated by a pathologist with an extensive experience in immunohistochemistry (K.W.). The staining intensity was rated on a scale of 0–4. The area of maximal intensity was used for grading as long as it comprised at least 10% of the region of interest. In general the staining was homogeneous without tremendous variations in staining intensity in the regions of interest. Therefore, quantitation of the proportion of cells staining was not undertaken.

Immunohistochemistry protocol for the detection of 13-S-HODE in human colorectal tissues

Staining was similar to that described for 15-LOX-1 with the following modifications: (i) non-specific antibody binding sites were blocked using 20% rabbit serum in PBS for 30 min; (ii) tissue sections were incubated in (1:6000) goat anti-13-S-HODE antibody solution at room temperature for 2 h; (iii) the secondary antibody solution was a 1:100 biotinylated anti-goat antibody solution (Vector Laboratories). In negative control experiments the primary antibody solution was substituted with PBS or goat serum. Frozen sections

containing cancerous and hyperplastic prostatic tissues were used as a positive control, because the anti-13-S-HODE antibody used in our study was reported previously to react with 13-S-HODE in human prostatic tissues (29).

Transformed colonic cell treatment with 13-S-HODE in vitro

RKO and HT-29 cells were plated in 60 mm tissue culture dishes (5×10^5 cells/dish for RKO and 1.4×10^5 cells/dish for HT-29) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin (Gibco BRL, Grand Island, NY). Serial dilutions of standard 13-S-HODE were made using PBS. 13-S-HODE was added to the medium on the next day of cell plating (pre-confluent conditions). Cells were incubated for 48 h in five culture dishes. To each culture dish one of the following serial dilutions of 13-S-HODE was added twice a day during 48 h incubation: (i) 0 μM (control); (ii) 17 μM; (iii) 34 μM; (iv) 67 μM; (v) 135 μM.

Cell cycle distribution analysis

Measurements of cell cycle were conducted 14 h after the last dose of 13-S-HODE (30). Cells were washed with cold PBS and fixed with ice-cold 70% ethanol in PBS at 4°C overnight. Fixed cells were washed twice with PBS, incubated with RNase type IIA (20 μg/ml) (Sigma, St Louis, MO) for 30 min at 37°C and then labeled with propidium iodide (50 μg/ml) (Sigma). DNA contents were measured with a Coulter ELITE flow cytometer. A multicycle software program (Phoenix Flow System, San Diego, CA) was used to produce histograms of DNA content frequency.

Cellular apoptosis

Apoptosis was measured using the following methods.
Flow cytometry analysis. Sub-diploid DNA peaks were quantified from the cell cycle distribution data to measure apoptosis.
Phase contrast microscopy. RKO and HT-29 cells were assessed in culture dishes prior to harvesting by phase contrast microscopy (Nikon) for morphological changes typical of apoptosis: cytoplasmic and nuclear shrinkage, chromatin condensation, membrane blebbing and formation of small apoptotic bodies leading to fragmentation of the cells (31,32).
Electron microscopy. Cultured RKO cells were pelleted and fixed with 2.5% glutaraldehyde in 0.1 M PBS for 24 h at 4°C. Cells were afterwards rinsed with PBS and post-fixed with 1% osmium tetroxide for 1 h, then dehydrated through a series of graded alcohol concentrations and, later, with propylene oxide. Cells were embedded in Epon resin. Ultrathin sections were cut, stained with lead citrate and uranyl acetate and inspected with a Phillips CM-100 transmission electron microscope.

DNA gel electrophoresis

Harvested RKO cells were suspended in 0.5 ml lysis buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1% SDS, 400 mM NaCl) (33). Cell lysates were incubated at 50°C for 2 h with 0.2 mg/ml proteinase K. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and re-extracted with chloroform/isoamyl alcohol and then precipitated with isopropanol at –70°C overnight. Precipitated DNA was centrifuged at 14 000 *g* for 30 min, washed with ice-cold 70% ethanol, resuspended in Tris–EDTA buffer (pH 8.0) and treated with 20 μg/ml DNase-free RNase (Boehringer Mannheim). DNA samples were applied to 1.6% agarose gels and visualized using ethidium bromide staining.

Statistical analysis

Data were analyzed using SAS software (SAS Institute, Cary, NC). Summary statistics are presented as the median and 95% confidence interval. 13-S-HODE values, measured by ELISA, were $\log_{10}$ transformed due to their non-normal distribution. Paired *t*-tests were used to test for significance of the differences between tumor and normal tissue levels ($\log_{10}$ transformed values). Differences in 13-S-HODE concentration were expressed as ratios of normal to tumor (mean and 95% confidence interval). 15-LOX-1 protein measurements were non-normally distributed, therefore, a non-parametric test (Wilcoxon signed rank test) was applied to compare levels among normal and tumorous tissues. Differences (normal – tumor) were expressed as median and 95% confidence intervals. Similarly, data for the immunohistochemical staining of 13-S-HODE and 15-LOX-1 had non-normal distributions (categorical data) and were analyzed analogously.

Results

The median age for the patients was 69.5 years (range 49–79) and the male to female ratio was 13:5 (2.6). Tumor characteristics are shown in Table I.

Tissue levels of 13-S-HODE as measured by ELISA

13-S-HODE levels were higher in the normal-appearing colon tissues than the tumor areas (normal, median 0.37 ng/μg protein, 95% CI 0.074–2.490; tumor, median 0.12 ng/μg protein, 95% CI 0.036–0.272). The mean difference between the normal and tumor tissues, expressed as the ratio normal: tumor, was >3 times higher in the normal tissues than tumor tissues (mean 3.33, 95% CI 1.28–8.677) and was statistically significant ($P = 0.0167$) (Figure 1).

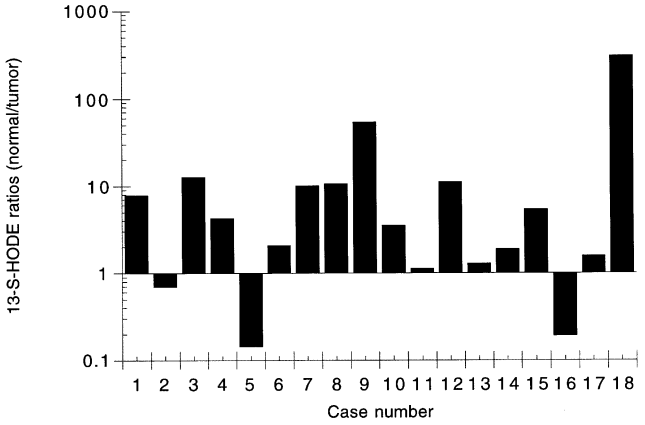

Fig. 1. Ratios of 13-S-HODE levels in paired tumor and normal colonic epithelia. 13-S-HODE levels were measured using an ELISA method and were normalized to protein content in each sample. The ratio of normal to tumor tissue concentrations was calculated for each patient and is represented by closed bars in the figure. Median ratio 3.33 (95% CI 1.28–8.677, $P = 0.0167$).

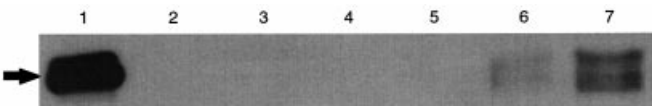

Fig. 2. 15-LOX-1 expression in malignant colon cell lines (HT-29, SW-620 and RKO), a normal endothelial cell line and human colon tissues. The arrow indicates the location of 15-LOX-1. Lane 1, standard recombinant human reticulocyte 15-LOX-1; lane 2, HT-29; lane 3, SW-620; lane 4, RKO; lane 5, negative control; lane 6, HUVEC; lane 7, normal colonic epithelial tissue (patient 14). Transformed colon cell lines did not express 15-LOX-1 (lanes 2–4), while 15-LOX-1 was detected in normal HUVEC (lane 6) and a normal human colonic tissue sample (lane 7) on the same western blot.

Table I. Tumor characteristics

	No. of patients
Tumor differentiation	
Poor	1/18 (6%)
Moderate	13/18 (72%)
Well	4/18 (22%)
Tumor location	
Rectum	2/18 (11%)
Sigmoid	8/18 (44%)
Transverse	1/18 (6%)
Right colon	5/18 (29%)
Multiple	2/18 (11%)

15-LOX-1 protein expression levels as measured by western blot

Immunoblots showed protein bands for 15-LOX-1 in the upper 70 kDa range, which corresponds to the electromigration area for human recombinant 15-LOX-1 (34; Figure 2). Standard human recombinant 15-LOX-1 (positive control) consistently showed the described bands on each gel, while no bands were seen in the negative control lanes. Although 15-LOX-1 protein expression in the tumors was higher than the normal areas

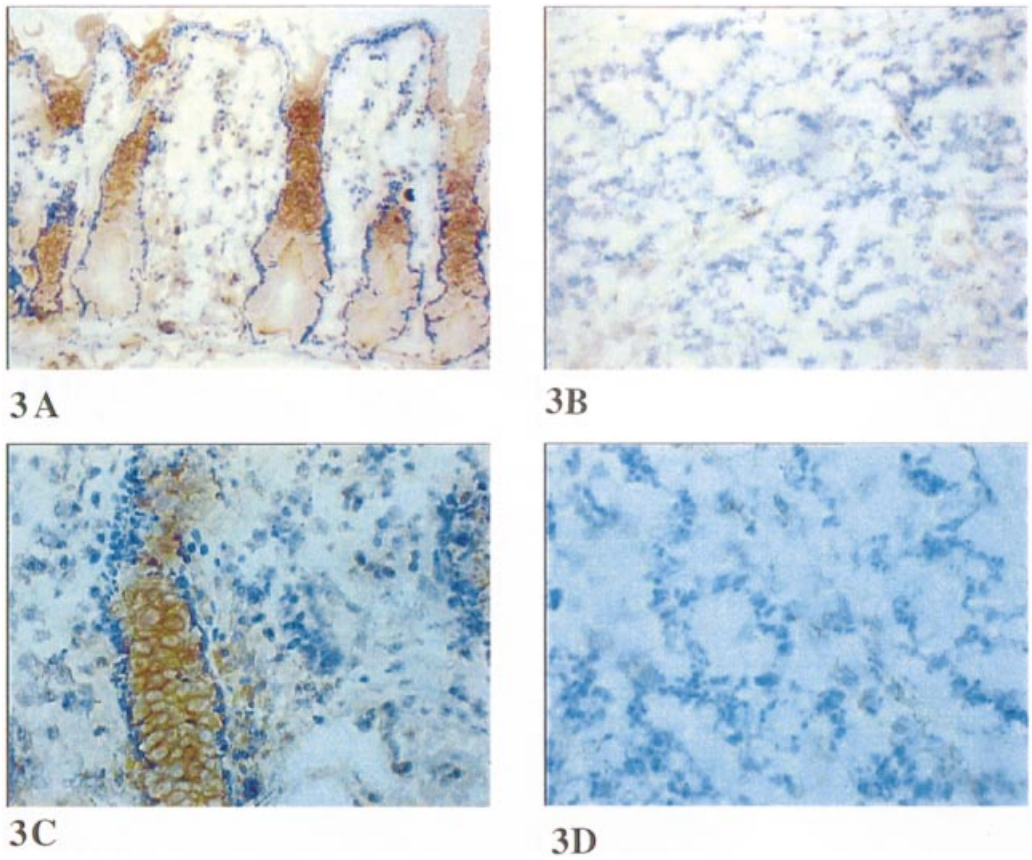

Fig. 3. 15-LOX-1 expression in human normal and cancer colonic tissues by immunohistochemistry. Immunohistochemical staining was performed on frozen tissue sections using an immunoperoxidase method. (**A**) A low magnification (200×) picture of a normal tissue section. (**B**) A tumor section from the same patient (200×). (**C**) A high magnification (400×) picture of the normal tissue section in (A). (**D**) A high magnification (400×) of the cancer tissue section in (B). Tumor epithelium had markedly decreased staining (brown color) when compared with normal epithelium.

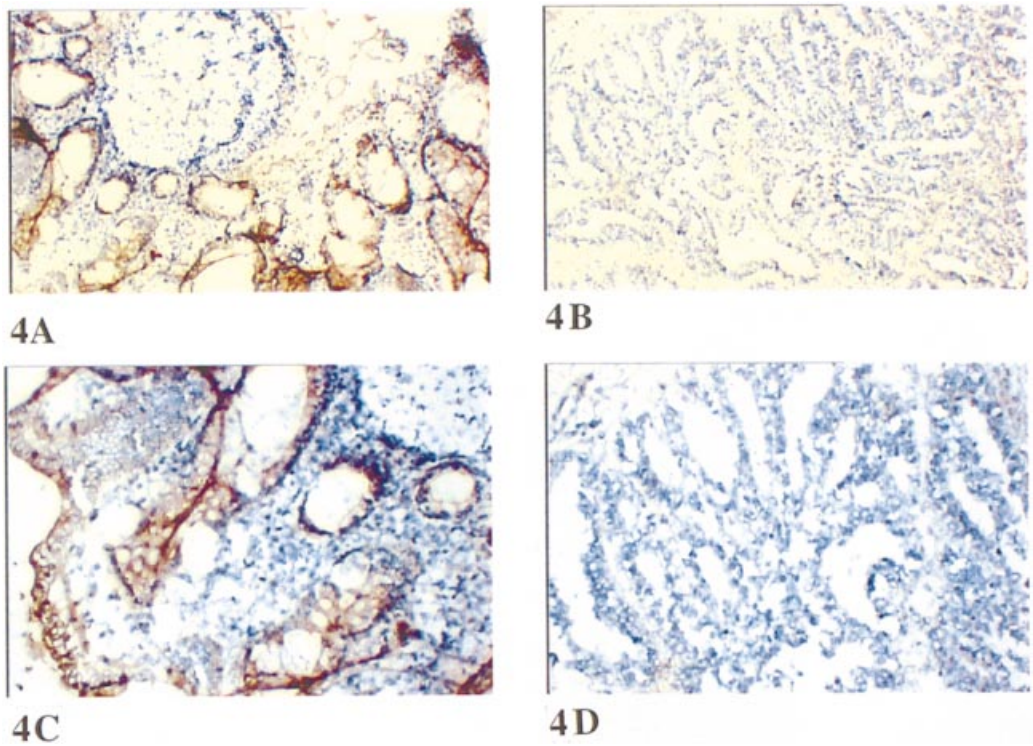

Fig. 4. 13-S-HODE expression by immunohistochemistry in human normal and cancer colonic tissues. Immunoperoxidase staining of frozen tissue sections was also used. (**A**) A low magnification (200×) picture of a normal tissue. (**B**) A low magnification (200×) picture of a cancer tissue section from the same patient. (**C**) A high magnification (400×) picture of the normal tissue section in (A). (**D**) A high magnification picture (400×) of the tumor tissue section in (B). 13-S-HODE staining (brown color) was reduced in colonic cancer epithelium.

(normal, median 16.39 ng/50 μg crude protein, 95% CI 10.49–29.1; tumor, median 26.86, 95% CI 14.28–33.20), the difference (normal – tumor) was not statistically significant (median –3.25, 95% CI –18.373–6.964, $P = 0.5296$). 15-LOX-1 protein was not detected by western blotting in three transformed colonic cell lines (HT-29, RKO and SW-620), while the 15-LOX-1 bands were seen on the same immunoblot for the 15-LOX-1 standard, samples from HUVEC and normal and tumor tissues (Figure 2).

15-LOX-1 expression by immunohistochemistry

Immunohistochemical staining was cytoplasmic in normal and tumor epithelia (Figure 3). Staining of lung tissue sections was positive for eosinophils and bronchial epithelial cells, as previously reported (data are not shown; 23). Staining was absent when the primary antibody solution was replaced by PBS or rabbit serum. 15-LOX-1 expression was markedly decreased in the tumor tissues as compared with their normal counterparts (normal, median 2.5; tumor, median 1.0) (Figure 4). The median difference between normal and tumor staining scores (normal – tumor) was equal to 2 and statistically significant ($P = 0.0001$).

13-S-HODE expression by immunohistochemistry

Immunohistochemical staining for 13-S-HODE was detected in the cytoplasms of normal and tumor epithelia (Figure 4). Prostatic tissues (positive control) showed positive staining, which was reduced in tumors compared with hyperplastic tissues (data not shown), while staining was absent when the primary antibody solution was replaced by PBS or goat serum. The immunohistochemical staining of 13-S-HODE was decreased in tumors, the difference (normal – tumor) being statistically significant (normal, median 3.0; tumor, median 2.0; difference, median 1, $P = 0.0001$).

13-S-HODE effects on RKO and HT-29 cell proliferation, cell cycle distribution and apoptosis

Given the unexpected findings of decreases in 13-S-HODE levels and 15-LOX-1 expression in human colon cancers, we next evaluated the *in vitro* effects of 13-S-HODE on transformed colonic cell lines (RKO and HT-29). 13-S-HODE decreased proliferation rates of RKO and HT-29 in a dose-dependent fashion, as shown in Figures 5 and 6. The proportion of floating/attached cells increased in a dose-dependent manner consistent with apoptosis induction in treated cells (Figure 7; 35). Phase contrast microscopic evaluation showed morphological changes typical of cell apoptosis (cytoplasmic and nuclear shrinkage, chromatin condensation, membrane blebbing and formation of small apoptotic bodies leading to fragmentation of cells) (31,32) in cells exposed to 13-S-HODE, as shown in Figure 8A for RKO cells (similar changes were observed in HT-29; data not shown). In contrast, linoleic acid at a concentration equal to the highest dose level used for 13-S-HODE (135 μM) caused no inhibition of cell proliferation rates (Figure 6) and no morphological evidence of apoptosis (Figure 8C). The proportion of cells that exhibited these morphological changes increased according to 13-S-HODE dose to involve all cells at a concentration of 135 μM. The fraction of cells in the sub-diploid peak (sub-G_1) increased in treated RKO and HT-29 cells in a dose-dependent fashion (47.3% at a concentration of 135 μM for RKO cells), which is indicative of apoptosis (36; Figure 9 and Table II). Furthermore, 13-S-HODE caused cell cycle arrest in RKO and HT-29 cells, which was evident from the decrease in the proportions of cells in the S and G_2 phases and the increase in the G_1/G_2 ratio (Tables II and III and Figure 10). At the highest dose level of 13-S-HODE, the fraction of cells in S phase rose again after a gradual drop at the lower concentrations. This paradoxical increase is likely because of S fraction 'contamination' with a marked increment in the number of apoptotic cells in the late S, G_2 or M phases (37). Apoptosis was confirmed in RKO cells by a DNA laddering assay which showed DNA fragmentation into a typical 'ladder' pattern of 200 bp integer multiples (38; Figure 11). RKO cells treated with 13-S-HODE showed morphological alterations by electron microscopy which are consistent with apoptosis: condensed and crescent-shaped chromatin associated with the nuclear membrane, cytoplasmic condensation and convolution of the cell surface and nuclear membrane (31,32; Figure 12).

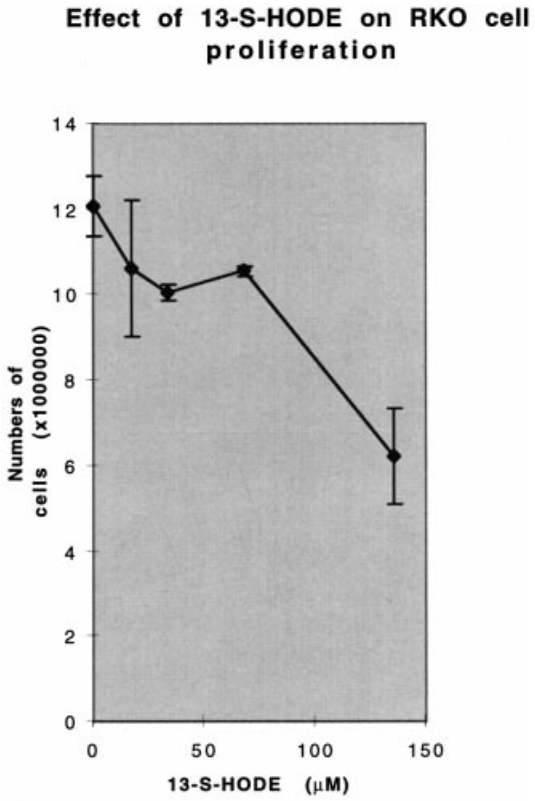

Fig. 5. 13-S-HODE effects on RKO cell proliferation rates. RKO cells were seeded at a concentration of 1.7×10^5 cell/ml and cells were treated twice a day for 48 h with one of a serial dilution of 13-S-HODE as shown on the *x*-axis. Fourteen hours after the last treatment, cells were harvested and counted. Attached cell counts are shown from duplicate experiments.

Discussion

Contrary to our expectations, we have found that 13-S-HODE levels are reduced in human colonic tumors. The average level of 13-S-HODE is >3-fold higher in normal colonic epithelium than in the tumor counterpart by ELISA measurement (Figure 1) and, similarly, 13-S-HODE immunohistochemistry staining scores are also reduced in tumors (Figure 4). In agreement with these results, colonic tumors have a marked decrease in expression of 15-LOX-1 by immunohistochemical staining (Figure 3) and 15-LOX-1 expression is absent in three transformed colonic cell lines. In contrast to transformed colonic cells, a HUVEC line, which was used for comparison because of the lack of a normal colonic cell line, did express

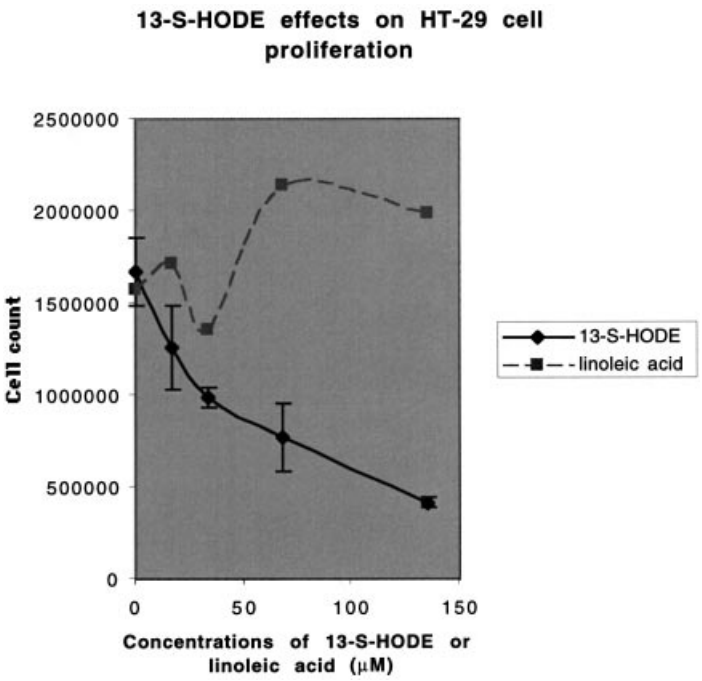

Fig. 6. 13-S-HODE effects on HT-29 cell proliferation rates. HT-29 cells were treated with one of a serial dilution of 13-S-HODE as shown on the *x*-axis twice a day for 48 h. Fourteen hours after the last treatment, cells were harvested and counted. Values shown are from duplicate experiments. Three other experiments showed similar results. Linoleic acid effects on HT-29 are shown in the upper curve. HT-29 cells were treated with linoleic acid using the same experimental conditions except for substituting 13-S-HODE with linoleic acid.

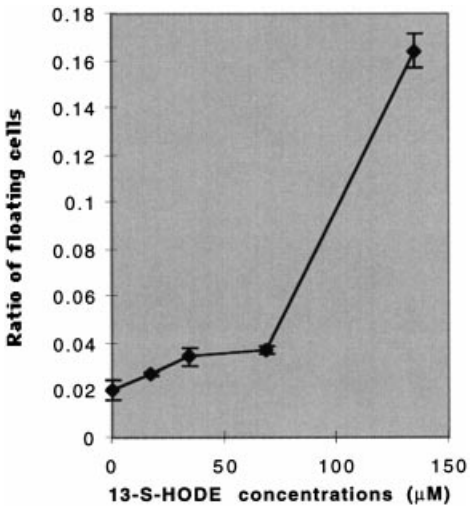

Fig. 7. 13-S-HODE increased the ratio floating:total cells in RKO cell cultures. RKO cells were treated with one of a serial dilution of 13-S-HODE as shown on the *x*-axis twice a day for 48 h. Fourteen hours after the last treatment, cells were harvested and counted. Values shown are from duplicate experiments.

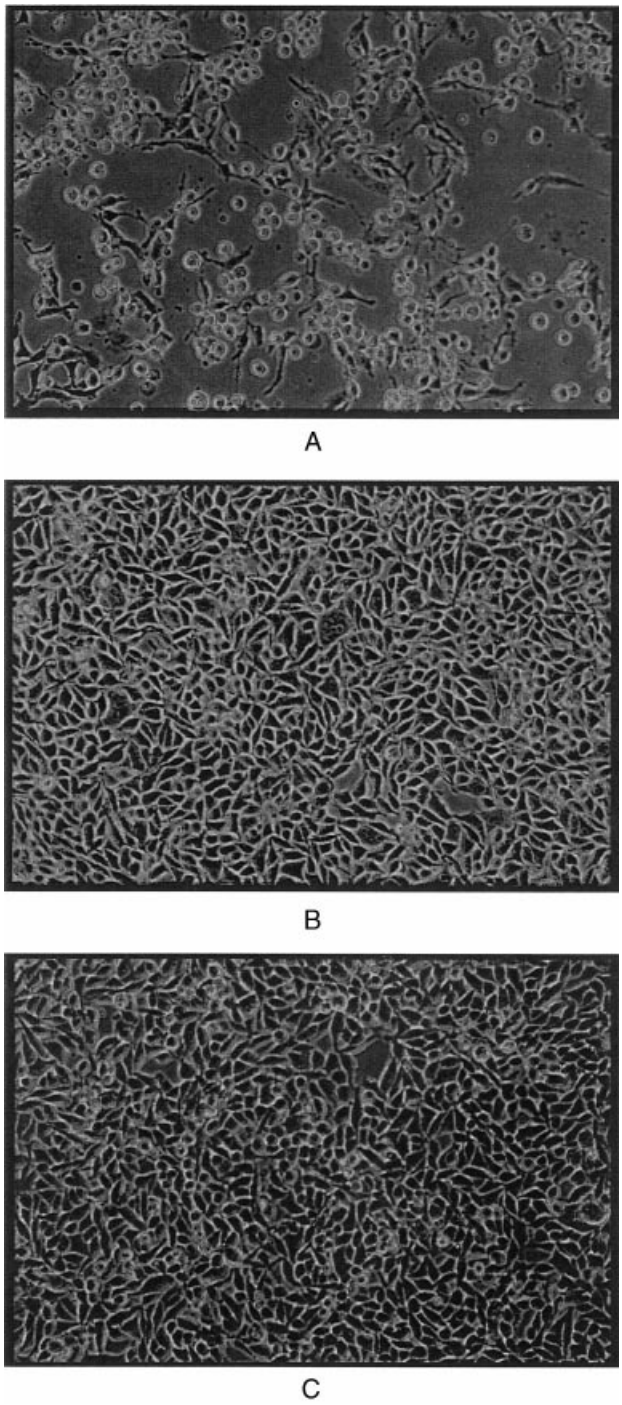

Fig. 8. 13-S-HODE inhibited cell proliferation and induced apoptosis in RKO cells. RKO cells were treated with 13-S-HODE twice a day for 48 h and pictures were obtained 14 h after the last treatment using a camera mounted on a phase contrast microscope. (**A**) RKO cells treated with 13-S-HODE at a concentration of 135 μM. Treated cells exhibited the typical morphological changes of apoptosis: cytoplasmic and nuclear shrinkage, chromatin condensation, membrane blebbing and formation of small apoptotic bodies leading to fragmentation of the cells. (**B**) A control experiment. (**C**) RKO cells treated with linoleic acid at a concentration of 135 μM (equal to the highest 13-S-HODE concentration used).

15-LOX-1 protein (Figure 2). All these findings are inconsistent with our initial hypothesis that 15-LOX-1 expression and 13-S-HODE levels are increased in human colon cancers. Rather, our data indicate that both 15-LOX-1 expression and 13-S-HODE levels are reduced in human colon cancers. One possible explanation for these results is that 13-S-HODE inhibits colonic epithelial proliferation and/or induces apoptosis, thus a loss of 13-S-HODE production through 15-LOX-1 down-regulation plays a role in colonic carcinogenesis. Alternatively, the observed decrease in 13-HODE might be an epiphenomenon to colonic carcinogenesis. The former explanation is more likely given our *in vitro* data showing that 13-S-HODE suppresses proliferation (Figures 5, 6 and 8) and induces cell cycle arrest and apoptosis in transformed RKO and HT-29

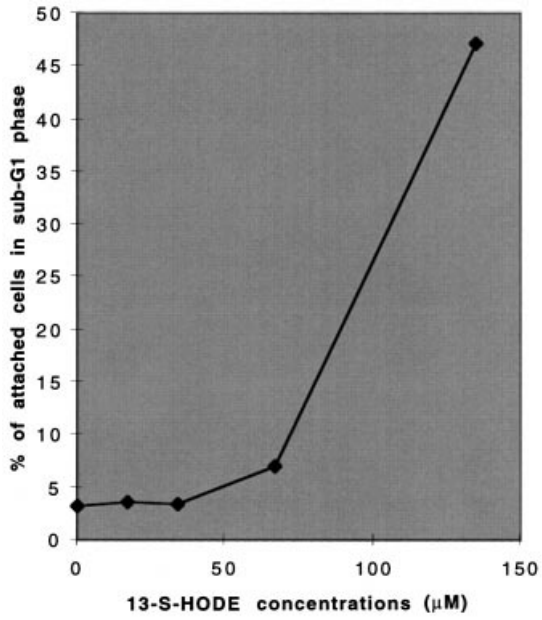

Fig. 9. 13-S-HODE increased the proportion of adherent RKO cells in sub-G_1 phase. Cells were treated with serial dilutions of 13-S-HODE and at the end of the incubation period DNA content was analyzed using a flow cytometer as described in Materials and methods. 13-S-HODE increases the proportion of cells in sub-G_1 phase in a dose-dependent manner.

Table II. Effects of 13-S-HODE on the cell cycle distribution of attached HT-29 cells

	G_0/G_1 (%)	S (%)	G_2/M (%)	G_1/G_2	Sub-G_1
13-S-HODE (μM)					
0	61.2	30.2	8.65	7.075	0.02
17	61.8	30.1	8	7.725	0.03
34	76.35	17.7	5.95	12.83	0.03
67	80.75	13.95	5.25	15.38	0.15
135	77.45	15.15	7.4	10.47	0.16

HT-29 cells were treated with 13-S-HODE twice a day for 48 h. Cells were harvested and fixed 14 h after the last treatment. Cells were labeled with propidium iodide and cell cycle distributions were determined by flow cytometry. Values represent the means. Experiments were repeated four times. In cells exposed to 13-S-HODE, the proportions of cells in S phase and the G_2/M phases decreased, while the percentage of cells in sub-G_1 and the G_0/G_1 and G_1/G_2 ratios increased, indicating that 13-S-HODE causes apoptotic cell cycle arrest in HT-29 cells. For the 135 μM concentration of 13-S-HODE the S phase fraction increased, after decreasing with lower concentrations of 13-S-HODE. This phenomenon is likely to be secondary to contamination of the S fraction with an increased number of apoptotic cells in the late S, G_2 or M phases.

colonic cells (Figures 7 and 9–12 and Tables II and III). Other observations in the literature support the concept that 13-S-HODE inhibits cell proliferation and induces apoptosis: First, when human 15-LOX-1 is expressed and metabolically active in human osteosarcoma cells, cell proliferation rates decrease by >50% (22). As 15-LOX-1 expression is lost, cell proliferation rates approach that observed in non-expressing clones. Second, 13-hydroperoxydecadienoic acid, an immediate precursor of 13-S-HODE, induces apoptosis in a human T cell line (A3.01) (39). Third, 13-S-HODE reverses skin hyperproliferation in guinea pigs (40) and the levels of 1-acyl-2,13-HODE-glycerol, a free intracellular form of 13-S-HODE, are inversely correlated with the degree of skin hyperproliferation (41). Fourth, 13-S-HODE attenuates ornithine decarboxylase activity and thus reduces polyamine-induced cellular proliferation in mucosal explants of rat colons (42). Finally, 13-S-HODE also contributes to the degeneration process of intracellular organelles such as mitochondria during the maturation of erythrocytes (43) and can conceivably act similarly during apoptosis in colonic cells. In addition, other data suggest that 13-S-HODE is unlikely to have tumorigenic effects: (i) differentiation of human tracheobronchial epithelial cells causes 15-LOX-1 expression (26); (ii) 13-S-HODE blocks 12-*S*-hydroxyeicosatetraenoic acid (12-S-HETE) from enhancing tumor cell adhesion to a variety of biological substrates (endothelial cells, sub-endothelial matrix and fibronectin) (20,44). 13-S-HODE is also unlikely to potentiate cellular proliferation even during the early steps of transformation, because 13-S-HODE reverses skin hyperproliferation in non-malignant skin lesions and suppresses ornithine decarboxylase activity in normal colonic explants. Taken together, our results and data from other laboratories argue against the concept that 13-S-HODE mediates the effect of linoleic acid in enhancing cellular proliferation. 13-S-HODE rather appears to inhibit cell proliferation and to be involved in cell apoptosis. Linoleic acid might promote tumorigenesis by other mechanisms, including the conversion of linoleic acid to arachidonic acid, which in turn can form several metabolites capable of enhancing the growth of transformed colonic cells (e.g. leukotriene B_4 and 12-R-HETE) (36).

The decrease in 13-S-HODE intracellular levels in tumors is unlikely to be secondary to an increased consumption of 13-S-HODE in tumor tissues. 13-S-HODE metabolism depends on the activity of the enzyme 13-HODE dehydrogenase (4) and an increase in 13-S-HODE consumption is expected to result in up-regulation of 13-HODE dehydrogenase activity. 13-S-HODE dehydrogenase activity is reduced in colon cancers (19), therefore, an increase in 13-S-HODE consumption in colonic tumors is unlikely. On the other hand, the reduction in 13-HODE dehydrogenase substrate, 13-S-HODE, in colonic cancer cells offers a new explanation for the decrease in 13-HODE dehydrogenase activity in colon cancer tissues.

We have found that 15-LOX-1 protein expression was lower in tumors than in normal tissues by immunohistochemistry. Furthermore, transformed colonic cell lines did not express 15-LOX-1 by western blot analysis, which further indicates that 15-LOX-1 is down-regulated in colonic tumors. No significant difference in 15-LOX-1 levels was detected by western blots between the normal and tumor tissue samples. Our inability to detect the difference by western blotting might have resulted from tumor samples containing a higher proportion of connective tissue cells. Tumor cells infiltrate into the sub-epithelium and are mixed with connective tissue and sometimes with inflammatory cells, which precludes a complete separation of tumor epithelial cells from connective tissue. 15-LOX-1 has been detected in fibroblasts, eosinophils, smooth muscle and blood vessel cells (12,23,24) and the presence of these cells in tumor samples is likely to have increased 15-LOX-1 levels. In contrast, immunohistochemistry can better define 15-LOX-1 expression in each tissue compartment and can distinctively reveal enzymatic expression in tumor and normal colonic epithelia. Additionally, immunohistochemical staining, in contrast to western blots, can reflect changes in 15-LOX-1 subcellular distribution, such as a reduction in 15-LOX-1 localization to the sub-membrane compartment. Studies in erythrocytes showed that 15-LOX-1 activation is associated with 15-LOX-1 enzymatic translocation

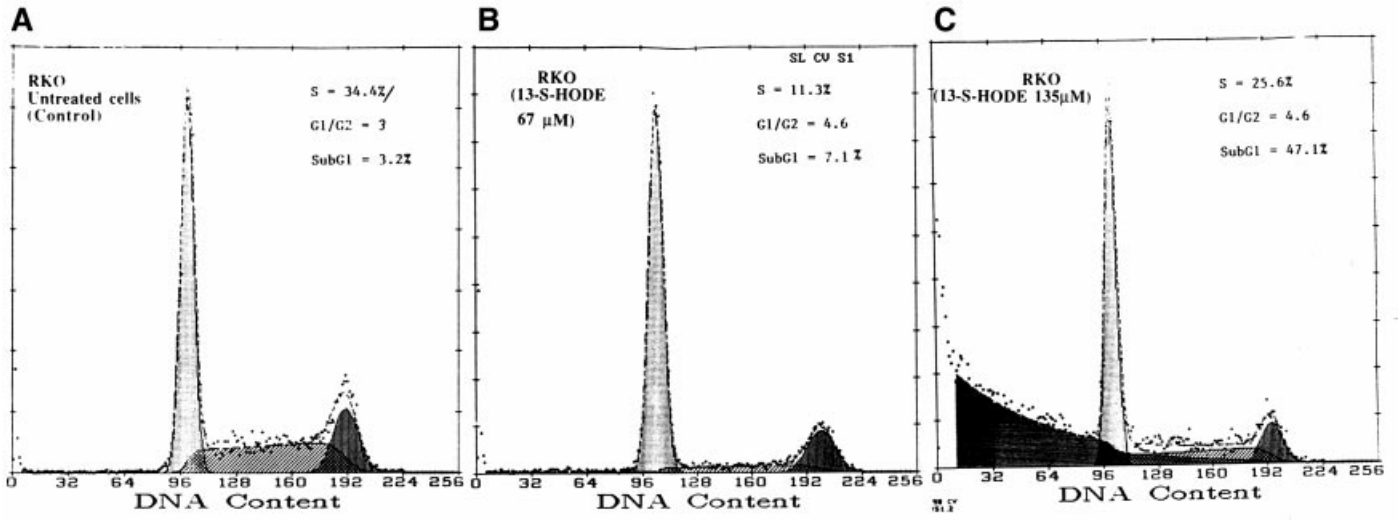

Fig. 10. Cell cycle distributions of RKO cells treated with 13-S-HODE. RKO cells were treated with 13-S-HODE twice a day for 48 h and cells were harvested 14 h after the last treatment. (**A**) Untreated cells (control); (**B**) 67 µM 13-S-HODE; (**C**) 135 µM 13-S-HODE. DNA contents were analyzed using propidium iodide staining as described in Materials and methods. The sub-G_1 phase area is represented by the area under the curve to the left of the G_1 peak on each histogram. The proportion of cells in S phase decreased while the G_1/G_2 ratio increased in treated cells compared with control cells which indicates that treated cells underwent cell cycle arrest**.**

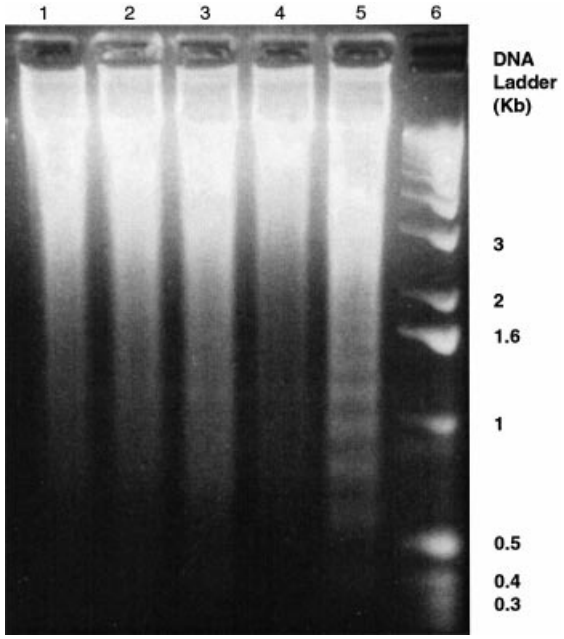

Fig. 11. 13-S-HODE induced DNA fragmentation in RKO cells. Cells were treated with one of a serial dilution of 13-S-HODE twice a day for 48 h and were harvested 14 h after the last treatment. DNA from treated and untreated (control) cells was prepared and analyzed by agarose gel electrophoresis as described in Materials and methods. Lane 1, cells from control experiment; lanes 2–5, cells treated with 13-S-HODE at 17 (lane 2), 34 (lane 3), 67 (lane 4) and 135 µM (lane 5); lane 6, DNA ladder.

to the sub-membrane compartment (45). Thus the reduction in tumor expression of 15-LOX-1 by immunohistochemical staining might also reflect a decrease in enzymatic activation, even if 15-LOX-1 protein levels were similar in the tumor and normal epithelia as measured by western blotting.

Recently, Ikawa *et al*. published a study that examined 15-LOX-1 expression in human colon cancers compared with paired normal tissue (12). They observed a relatively small increase in 15-LOX-1 protein expression in the tumor tissues by western blot using 'crude' tissue homogenates without dissecting epithelial from stromal tissues. The observed increases in 15-LOX-1 protein levels in tumor samples could be the result of higher contamination of tumor samples with stromal tissues. Tumors tend to invade the stromal compartment and therefore tumor samples have a higher probability of stromal contamination than normal paired samples. Ikawa *et al*. used an immunohistochemical method to localize 15-LOX-1 expression to the epithelium. 15-LOX-1 expression was detected in both the tumor and normal epithelial tissues and in fibroblasts located within the stromal tissues. They 'could not make a quantitation of expression between tumor and adjacent normal tissue by the immunohistochemical method'. This leads us to conclude that they were unable to detect a difference to support their conclusion that 15-LOX-1 is up-regulated in the tumors. One possible explanation for not detecting the differences is that the titer of antibody used was too high to allow detection of a difference between the normal and tumor tissues (Ikawa *et al*. used a paraffin-embedded section with a 15-LOX-1 antibody titer of 1:200, compared with 1:8000 in our study using the same antibody source). Thus the data presented in Ikawa's study are insufficient to confirm that 15-LOX-1 is up-regulated in colonic tumor epithelial tissues. Based on the result of our 15-LOX-1 immunohistochemical measurements, the lack of 15-LOX-1 expression in several transformed colonic cell lines and our findings of reduced 13-S-HODE levels in tumors by ELISA and immunohistochemistry, we feel that the preponderance of evidence supports the concept of 15-LOX-1 being down-regulated in human colon cancers.

Kamitani *et al*. have found no expression of 15-LOX-1 in a transformed colonic cell line (Caco-2) (25), similar to our finding in three other transformed colonic cell lines (Figure 2). Sodium butyrate (NaBT) induced Caco-2 cell differentiation and apoptosis that were associated with 15-LOX-1 expression. As cells progressed through this process of differentiation and apoptosis they acquired the ability to form 13-S-HODE when incubated with linoleic acid. Nordihydroguaiaretic acid (NDGA), a non-specific lipoxygenase inhibitor, increased NaBT-induced apoptosis in Caco-2 cells. As an explanation,

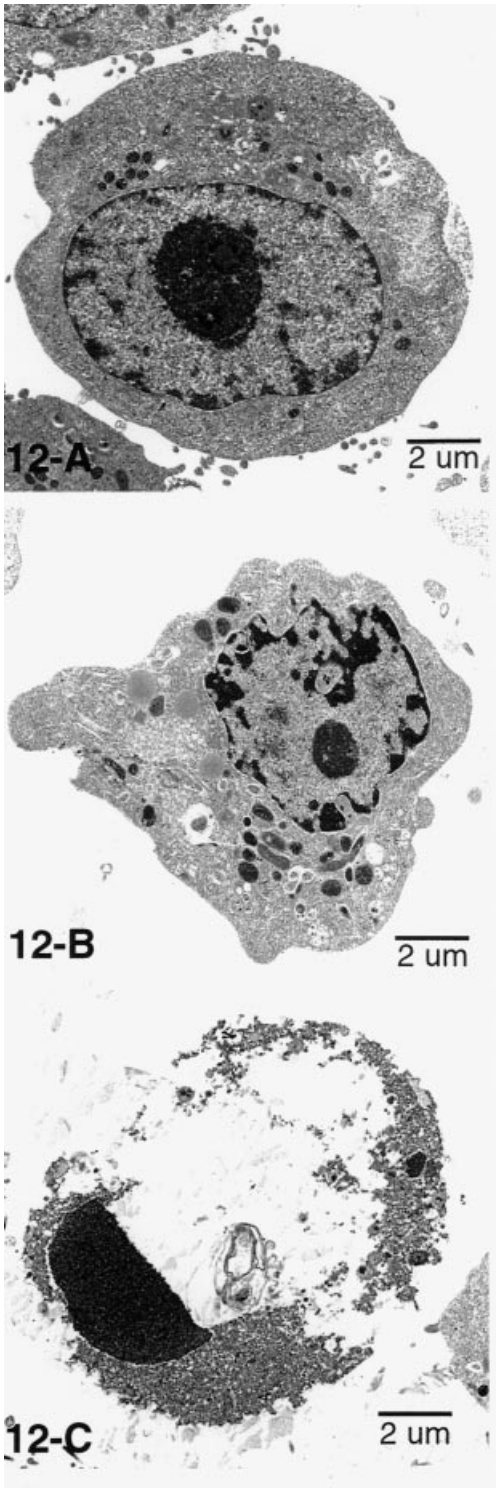

Fig. 12. Electron microscopic images of RKO cells treated with 135 μM 13-S-HODE (B and C) and untreated cells (A). (**A**) RKO cell from a control experiment showing normal appearance at the end of the incubation period. (**B**) RKO cell exposed to 13-S-HODE and harvested at the same time as the cell in (A). The cell exhibits features typical of early stage apoptosis: (i) compaction and segregation of the condensed chromatin that adjoins the inner surface of the nuclear membrane; (ii) convolution of the nuclear envelope; (iii) condensation of the cytoplasm; (iv) convolution of the cellular membrane. (**C**) A partly degraded cell in a late stage of apoptosis with a condensed crescent-shaped chromatin fragment.

Table III. Effects of 13-S-HODE on the cell cycle distribution of attached RKO cells

	G_0/G_1 (%)	S (%)	G_2/M (%)	G_1/G_2
13-S-HODE (μM)				
0	49.5	34.4	16.2	3.06
17	55.5	30.3	14.3	3.88
34	59.6	26.8	13.7	4.35
67	72.8	11.3	15.9	4.58
135	61.1	25.6	13.4	4.56
Linoleic acid (135 μM)	51.1	24.6	24.3	2.1

RKO cells were treated with 13-S-HODE twice a day for 48 h. Cells were harvested and fixed 14 h after the last treatment. Cells were labeled with propidium iodide and cell cycle distributions were determined by flow cytometry. In cells exposed to 13-S-HODE, the proportions of cells in S phase and the G_2/M phases decreased, while the G_0/G_1 and G_1/G_2 ratios increased, indicating that 13-S-HODE causes cell cycle arrest in RKO cells. For the 135 μM concentration of 13-S-HODE the S phase fraction increased, after decreasing with lower concentrations of 13-S-HODE. This phenomenon is likely to be secondary to contamination of the S fraction with an increased number of apoptotic cells in the late S, G_2 or M phases. In contrast to 13-S-HODE, linoleic acid at a concentration of 135 μM raised the G_2 fraction and decreased the G_1/G_2 ratio.

Kamitani *et al*. proposed 'the hypothesis for 15-LOX-1 metabolites acting as inhibitor of apoptosis', however, they presented no data to show specific inhibition of 15-LOX-1 activity and 13-S-HODE formation during these experiments. They later stated that this interpretation 'must be viewed with caution', because 'NDGA is a general inhibitor of lipoxygenase and can influence the redox state of cells' (25). They have also mentioned that because a 'considerable amount of 13-HODE was produced from the cell lysates from NaBT-treated cells during cell differentiation and apoptosis . . . we must also consider if these lipids are modulators of differentiation'. As we have mentioned earlier, other lipoxygenase products, such as 12-HETE and leukotriene B_4, promote colonic cell proliferation. The use of a general lipoxygenase inhibitor is therefore inadequate to evaluate the role of 15-LOX-1 and 13-HODE in cell differentiation and apoptosis. One approach to evaluate this role is to test the direct effects of 13-S-HODE on colonic cells, which we examined in our *in vitro* study.

We found that 13-S-HODE induced apoptosis in RKO cells in a dose-dependent fashion, which became very strongly evident at the highest dose level used (135 μM) by the increase in the fraction of cells demonstrating sub-diploid (sub-G_1) DNA contents (Figure 9) and the proportion of floating/attached cells (Figure 7). Apoptosis was confirmed by DNA laddering (Figure 11) and electron microscopy (Figure 12). Additionally, 13-S-HODE caused cell cycle arrest, which has been shown to be temporally related to the onset of apoptosis (37). In contrast, linoleic acid at a concentration equal to the highest concentration of 13-S-HODE used had opposite effects on cell cycle distribution (Table III). Similarly, 13-S-HODE inhibited cell proliferation of HT-29 transformed colonic cells (Figure 6). 13-S-HODE also caused morphological changes, cell cycle arrest and an increase in the sub-G_1 phase, indicative of apoptosis (Table II). These observations show that 13-S-HODE effects are not limited to the RKO cell line. The levels of 13-S-HODE applied exogenously in our study might appear too high in comparison with the levels of some cytotoxic agents which can induce apoptosis *in vitro*. However, other agents, such as sulindac and sulindac sulfide, require compar-

able concentrations to the 13-S-HODE concentrations used in our study to trigger apoptosis in transformed colonic cells (sulindac, 120 μM; sulindac sulfone, 240 μM) (46). Furthermore, 13-S-HODE exists in normal resting cells at a concentration of 3410 ± 340 ng/10^6 cells, as measured in HUVEC (47). Currently, the intracellular levels of 13-S-HODE in normal colonic cells *in vitro* is unknown, because no normal colon cell line is available. If the levels of 13-S-HODE in resting HUVEC is used as an approximation to that expected in normal colonic cells, a 13-S-HODE concentration of 57 μM in the culture medium is required to reach that level. 13-S-HODE levels are expected to increase above the levels in resting cells for 13-S-HODE to activate apoptosis. In our *in vitro* studies the exogenous application of 13-S-HODE activated apoptosis markedly in RKO cells at concentrations <2.5-fold of the level in resting HUVEC. Additionally, 13-S-HODE is considered to be relatively unstable in culture media and sensitive to common elements such as light and oxygen which limit cell exposure to this compound when applied exogenously (Cayman Chemical Catalog, Vol. VIII, 1996). Therefore, the observed effects of 13-S-HODE are unlikely to have resulted from using concentrations that are far from physiological levels. Finally, in control experiments linoleic acid, the parent compound of 13-S-HODE, failed to inhibit the growth of RKO and HT-29 cells or induce cell cycle arrest (Figures 6 and 8C) at a concentration equal to the highest 13-S-HODE concentrations used in our study (135 μM). This further indicates that a non-specific fatty acid toxicity is unlikely to have produced the 13-S-HODE effects.

In summary, our study demonstrates that 15-LOX-1 protein expression and 13-S-HODE intracellular levels are decreased in human colonic tumors and that 13-S-HODE inhibits cell proliferation and induces cell cycle arrest and apoptosis in RKO transformed colonic cells. These findings contradict the notion that 13-S-HODE promotes colonic carcinogenesis and indicate that 13-S-HODE might have a role in the inhibition of colonic cell proliferation and the induction of apoptosis.

Acknowledgements

We thank Dr Alnawaz Rehemtulla and Joseph Cornicelli for reviewing the manuscript and for their helpful scientific comments and Daniel Cutler for his efforts in preparing the immunohistochemistry pictures. This work has been supported in part by an institutional grant from the American Cancer Society (ACS374639).

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Received February 26, 1999; revised June 8, 1999; accepted June 25, 1999