Reduction of Inflammatory Bowel Disease-Induced Tumor Development in IL-10 Knockout Mice With Soluble Epoxide Hydrolase Gene Deficiency

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Soluble epoxide hydrolase (sEH) quickly inactivates anti-inflammatory epoxyeicosatrienoic acids (EETs) by converting them to dihydroxyeicosatetraenoic acids (DHETs). Inhibition of sEH has shown effects against inflammation, but little is studied about the role of sEH in inflammatory bowel disease (IBD) and its induced carcinogenesis. In the present study, the effect of sEH gene deficiency on the development of IBD-induced tumor development was determined in IL-10 knockout mice combined with sEH gene deficiency. Tumor development in the bowel was examined at the age of 25 wk for male mice and 35 wk for female mice. Compared to IL-10(−/−) mice, sEH (−/−)/IL-10(−/−) mice exhibited a significant decrease of tumor multiplicity (2 ± 0.9 tumors/mouse vs. 1 ± 0.3 tumors/mouse) and tumor size (344.55 ± 71.73 mm³ vs. 126.94 ± 23.18 mm³), as well as a marked decrease of precancerous dysplasia. The significantly lower inflammatory scores were further observed in the bowel in sEH(−/−)/IL-10(−/−) mice as compared to IL-10(−/−) mice, including parameters of inflammation-involved area (0.70 ± 0.16 vs. 1.4 ± 0.18), inflammation cell infiltration (1.55 ± 0.35 vs. 2.15 ± 0.18), and epithelial hyperplasia (0.95 ± 0.21 vs. 1.45 ± 0.18), as well as larger ulcer formation. qPCR and Western blotting assays demonstrated a significant downregulation of cytokines/chemokines (TNF-α, MCP-1, and IL-12, 17, and 23) and NF-κB signals. Eicosanoid acid metabolic profiling revealed a significant increase of ratios of EETs to DHETs and EpOMEs to DiOMEs. These results indicate that sEH plays an important role in IBD and its-induced carcinogenesis and could serve as a highly potential target of chemoprevention and treatment for IBD. © 2012 Wiley Periodicals, Inc.

Key words: inflammatory bowel disease; carcinogenesis; soluble epoxide hydrolase; IL-10; eicosanoid acid metabolic profiling

INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease, is a longstanding chronic active inflammatory process with an increased risk for the development of cancer in the intestinal tract. Ablation of effects of arachidonic acid in the process of long-term inflammation play a crucial role in inflammation-induced carcinogenesis [1,2]. Three key metabolic pathways are involved in arachidonic acid metabolism, including cyclooxygenase (COX)-mediated prostaglandins, lipoxygenase (LOX)-mediated leukotrienes and hydroxyeicosatetraenoic acids (HETEs), and cytochrome P450-mediated epoxyeicosatrienoic acids (EETs) [1]. There are numerous studies that indicate the COX- and LOX-mediated products promoting carcinogenesis [2]. But the third pathway of P450-mediated EETs is under study.

The role of P450-EETs-soluble epoxide hydrolase (sEH) pathway in inflammation has been assessed in rodent models [3–9]. The first finding for anti-inflammatory activity by EETs was reported in 1999 and showed that physiological concentrations of EETs or overexpression of p450-2J2 (that transformed arachidonic acid to EETs) inhibits inflammation through decreasing tumor necrosis factor-α (TNF-α)-induced vascular cell adhesion molecule expression (VCAM-1) and reducing leukocyte adhesion and infiltration to the vascular wall and inflamed tissues, and suppresses transcription factor NF-κB and IKK Kinase [10]. Under physiologic condition, EETs are quickly inactivated by sEH that converts them to their corresponding dihydroxyeicosatetraenoic acids (DHETs) [7]. It has been demonstrated that sEH inhibition or gene knockout stabilizes EETs and increases EETs to DHETs ratio [11,12], resulting in dramatically anti-inflammatory.

Abbreviations: IBD, inflammatory bowel disease; COX, cyclooxygenase; LOX, lipoxygenase; HETEs, hydroxyeicosatetraenoic acids; EETs, epoxyeicosatrienoic acids; sEH, soluble epoxide hydrolase; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule; DHETs, dihydroxyeicosatetraenoic acids; LC/MS-MS, liquid chromatography/mass spectrometry; MPO, myeloperoxidase; IFN, interferon; MCP-1, monocyte chemoattractant protein-1; PGE2, prostaglandin E2; TXB2, thromboxane B2; LTβα, leukotriene B4; 5-HETE, 5-hydroxyeicosatetraenoic acid; MCP-1, monocyte chemoattractant protein-1.

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function [3–9,13]. Using LPS-induced systematic inflammation in mice, treatment with sEH inhibitor reduces the production of nitric oxide, cytokines, and proinflammatory lipid mediators and significantly improves survival [7,14]. In the smoking induced bronchitis model in rats, sEH inhibitor shows a significant decrease of total bronchoalveolar inflammatory cells by 37% in tobacco smoke-exposed rats [8]. A combination of sEH inhibitor and EETs exhibits more significant effect on reducing tobacco-smoking-induced inflammation [8]. sEH knock-out mice (disruption of Epx2 gene which encodes sEH) are phenotypically normal with only minimally decreased body weight in males, but have an altered arachidonic acid metabolism [15]. sEH knockout in mice results in a significant shift of the epoxy-fatty acid to diol ratio, led to protection against myocardial ischemia-reperfusion injury, modulation of the inflammatory response to cerebral ischemia, and improvement of glucose homeostasis [16–18]. However, there is no study on investigating the role of sEH gene deficiency in long-term inflammation-induced carcinogenesis.

A spontaneous IBD and its induced carcinogenesis in IL-10 knockout [IL-10(−/−)] mice serves as a representative model of IBD [2]. Typically, IL-10(−/−) mice spontaneously develop chronic enterocolitis with the transmural inflammation pattern in the small and large bowel which is phenotypically mimicking human Crohn’s disease [19]. But the occurrence of IBD greatly varies from 3 to 12 months. Synchronizing the development of IBD has been achieved through the short-period administration of piroxicam (1-wk treatment) [20]. Development of cancer in intestinal tract, mainly in the cecum and small and large bowel which is phenotypically mimicking human Crohn’s disease [19]. But the occurrence of IBD greatly varies from 3 to 12 months. Synchronizing the development of IBD has been achieved through the short-period administration of piroxicam (1-wk treatment) [20]. Development of cancer in intestinal tract, mainly in the cecum and proximal small bowel, has been observed in IL-10(−/−) mice with long-term IBD [21].

Our short-term animal experiment has showed that sEH gene deficiency in IL-10(−/−) mice results in a significant attenuation of inflammatory activity in the gastrointestinal tract (published separately). In the present study, development of tumors and dysplasia in intestinal tracts was analyzed histopathologically and immunohistochemically in the long-term survival IL-10(−/−) mice and double knockout IL-10(−/−)/sEH(−/−) mice. Inflammation activity and its correlation with tumor development were investigated. qPCR and Western blotting assays were performed to analyze key inflammatory cytokines/chemokines and NF-kB signals. The eicosanoid metabolic profile was analyzed using a liquid chromatography/mass spectrometry (LC/MS-MS) method.

MATERIALS AND METHODS

Animal Breeding and Genotyping

All animal experiments were approved by the Institutional Animal Care and Use Committee at Northwestern University. IL-10(−/−) mice in a C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, ME), and sEH(−/−) mice in a C57BL/6J background were provided by Dr. Hammock at the University of California in Davis, CA. To produce homozygous double knockout sEH(−/−)/IL-10(−/−) mice, female IL-10(−/−) mice were first crossed with male sEH(−/−) mice to generate IL-10(+/−)/sEH(+/−) double heterozygous litters (F1 colonies). Then, sEH(−/−)/IL-10(−/−) mice were produced by further cross-breeding IL-10(+/−)/sEH(+/−) mice (F2 colonies). Genotyping was performed based on the protocol from Jackson Laboratory. All mice were housed in micro-isolator cages (3–5 mice/cage) in a room illuminated with 12-h/12-h light-dark cycle and with free access to water and chow in the animal facilities at the Center for Comparative Medicine at Northwestern University (Chicago, IL).

Animal Experiments and Tissue Processing

IL-10(−/−), sEH(−/−)/IL-10(−/−), sEH(−/−), and C57BL/6J wild-type mice (equal number of each gender per group) were used. Since the occurrence of IBD varies greatly in IL-10(−/−) mice, all mice at the age of 4 wk were treated with 200 ppm piroxicam in AIN 93M diet for a week. At age 5 wk, AIN 93M diet was administered to the mice until the end of experiment. Food and water consumption were monitored daily, and body weights were recorded every week. For examination of the tumor development, male mice were scarified at age 25 wk due to the exhibition of more than 20% loss of body weight; and female animals were sacrificed at age 38 wk.

Blood and plasma were collected via heart acupuncture and stored in a −80°C freezer until analysis. All of the key organs including entire gastrointestinal tract, liver, spleen, kidney, etc were collected. Briefly, small and large intestines were inflated by in situ intra-luminal perfusion with chilled normal saline solution and opened longitudinally. The number, size and distribution of intestinal lesions (ulcer and tumor) were determined. Tumor volume was determined using the equation $V = \frac{4}{3}\pi r^3$, where $r$ was the average tumor radius obtained from the two diameter measurements. Mucosal tissue from half of entire gastrointestinal tract was collected freshly in RNA later for biochemical assay including protein extraction for Western blot and RNA extraction for qPCR. The other half of the entire gastrointestinal tract (including stomach, small bowel and colon) were fixed in a 10% buffered formalin, prepared as “Swiss” rolls, and processed for paraffin sections for histopathological and immunohistochemical analyses.

Histopathological Analysis

Intestinal tumor was analyzed based on the established criteria [22,23]. Adenocarcinoma was diagnosed by the invasion of neoplastic cells through
the basement membrane into laminar propria and submucosa, and further classified as polyloid well, moderate, or poorly differentiated pattern based on formation of glandular structure and mucinous carcinoma with mucin production in more than 75% tumor. Dysplasia was characterized by the partial loss of cell polarity and maturation, nuclear atypia, and an increase in mitotic figures [24].

The grading of IBD was based on the established criteria, including the parameters of: (1) the portion of area involved by inflammation, (2) intensity of inflammatory cell infiltration, (3) reactive/regenerative epithelial hyperplasia, and (4) ulcer formation [22]. Briefly, the inflammation involved area was scored from 0 to 3: 0: no inflammation in intestine 1: <25% of intestine involved by inflammation; 2: 25–50%; and 3: more than 50%. The inflammatory cell infiltration was scored from 0 to 3: 0, minimal inflammatory cell infiltration in the mucosa of intestinal tract, mainly are plasma cells and lymphocytes; 1: focal intense inflammatory cell infiltration (<30% of inflammatory cells in total cells counted in the inflamed areas) with occasional neutrophils (<10% of inflammatory cells are neutrophils highlighted by myeloperoxidase immunostaining); 2: focal highly intense inflammatory cells (30–60% inflammatory cells in the inflamed areas with 10–20% myeloperoxidase (MPO)-positive-stained neutrophils) and epithelial injury and mucosal erosion, and 3: ulcer formation with intense and more than 60% inflammatory cells infiltration (>20% MPO-positive-stained neutrophils) in the inflamed areas. Epithelial hyperplasia was scored as 0–3 according to the ratio of the mucosal thickness to the entire intestinal wall, 0: the ratio is less than 1/3; 1: 1/3–1/2; 2: 1/2–3/4; and 3: more than 3/4. The score for ulcer was 0: no ulcer and 1: with ulcer formation. The total inflammation index will be the sum of all parameters.

**Immunohistochemistry**

Immunohistochemical staining was performed using avidin–biotin–peroxidase complex method on paraffin-embedded intestinal sections as previously described [22]. The primary antibodies are myeloperoxidase antibody (Rabbit pAb, 60 μg/μL, Abcam, Cambridge, MA) and Ki-67 antibody (Rabbit mAb, 1:100 diluted, Vector Laboratories, Inc., Burlingame, CA). Biotinylated anti-rabbit IgG secondary antibody (1:200) and ABC complex (avidin–biotin complex) were purchased from Vector Laboratories, Inc. Diaminobenzidine (Sigma-Aldrich Co., St. Louis, MO) was used as the chromogen. Slides were washed with 1× TBST buffer between incubations and counterstained with Mayer's hemotoxylin for 1½ min. The number of myeloperoxidase-positive-stained inflammatory cells per high power (40× objective lens) was counted for each specimen. Cell proliferation was analyzed by Ki-67-labeled cells and proliferation index was the percentage of Ki-67-positive cells in the total number of cells counted.

**Quantitative Real-Time PCR**

Total RNA was extracted from the colonic mucosa using the RNeasy kit (Qiagen, Inc., Valencia, CA). The concentration was determined using a SmartSpec Plus Spectrophotometer (BioRad, Hercules, CA). First-strand cDNA was synthesized using 1 μg of total RNA in a 20 μL reverse transcription reaction mixture using SuperScript III Platinum Two-Step q-RT-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The region of IFN-γ, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) mRNA were amplified using primers: IFN-γ (Forward 5′-TCA-AGTGGCATAGTTGGAAAGA; Reverse 5′-TGGCTCTGCAGAGATTTCTATG), TNF-α (Forward 5′-CCAA CGGCAATGTCTCTCAAGACA; Reverse 5′-TGGAGTAGCAAATCCGCTGAGC), MCP-1 (Forward 5′-ACTTAAGCCAGC TCTCTTCTCC; Reverse 5′-TTCTTCTTTGG GTCAAGCACAGA) as described in literatures [25,26]. The region of IL-12, IL-17, and IL-23 mRNA were amplified using primers IL-12 (Forward 5′-CAGATAGCCCATCACCCTGT; Reverse 5′-ACGGCCAGAGAAAACCTGAA), IL-17 (Forward 5′-TCTCTGATGCTTGCTGCT; Reverse 5′-CTGGTGAACCGTGTAGTGTG), and IL-23 (Forward 5′-AAATATGTCGCCGTATCCA, Reverse 5′-CTGGAGGAGTTGGCTGAGTC). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as internal control (Forward 5′-GCACAGTCAAGGGCGGAAAT; Reverse 5′-GCCCTTCTCTCATGGTGGTGA). All real-time PCR reactions were performed in a 25 μL mixture containing 1/10 volume of cDNA preparation (2.5 μL), 12.5 μL SYBR Green buffer (Invitrogen), 0.5 μM of each primer, 8.5 μL DEPC (diethylprocarboxylate) (EMD Chemicals, Gibbstown, NJ). Real-time quantitation was performed using the MiniOpticon Real-Time PCR System (BioRad). PCR conditions were: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C, 15 s; 58°C, 3 s; 50°C, 1 s. Data of each mRNA expression were shown as the relative fold of change normalized by that of GAPDH.

**Western Blotting**

One hundred fifty microliters of whole tissue/cell lysate of intestinal mucosa (n = 3 mice per group) was separated by 10% SDS-PAGE and protein expression was analyzed using the following antibodies: rabbit anti-NF-κB mAb P65 and phosphorlated NF-κB p65 (S276) mAb (1:1,000, Cell Signaling Technology, Danvers, MA), rabbit anti-IKK-α (1:1,000, Cell Signaling Technology), goat anti-VCAM-1 IgG Ab (1:750, R&D System, Minneapolis, MN), and mouse anti-β-actin mAb (1:2000, Sigma-Aldrich Co.). Signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).
Analysis of Arachidonic Acid Metabolic Profile Using LC/MS-MS Method

Serum specimens were spiked with 10 μL of 50 nM internal standard I (d4-6-keto-PGF1α, d4-PGF2α, d4-TXB2, d4-LTB4, d11-14,15-diHETE, d4-9-HODE, d8-5-HETE, d11-11(12)-EpiETE) and extracted by solid phase extraction using Oasis HLB cartridges (3 cm³ 60 mg, Waters, Milford, MA). The HLB cartridges were first washed with 2 mL ethyl acetate, 2 mL methanol twice, and 2 mL 95:5 (v/v) water/methanol with 0.1% acetic acid. Then, 200 μL serum samples were loaded onto the cartridges, washed with 6 mL 95:5 (v/v) water/methanol with 0.1% acetic acid, and dried for 20 min with low vacuum. The target analytes were eluted with 0.5 mL methanol, followed by 2 mL of ethyl acetate into the tubes with 6 μL 30% glycerol in methanol as the trap solution. The volatile solvents were evaporated using vacuum centrifugation (Speed-Vac) until 2 μL trap solution remained in the tube. The residues were dissolved in 50 μL of methanol containing 200 nM internal standards II (1-cyclohexyl-dodecanoic acid urea, CUDA). The samples were mixed with a vortex mixer for 2 min, centrifuged at 14 000g for 5 min and then transferred to auto sampler vials with 150 μL inserts for LC/MS-MS analysis.

Liquid chromatography/tandem mass spectrometry (LC/MS-MS) analysis of oxylipids was performed using a modified method based on the previous publication [27]. An Agilent 1200 SL liquid chromatography series (Agilent Corporation, Palo Alto, CA) with an Agilent Eclipse Plus C18 2.1 mm × 150 mm, 1.8 μm column were used for the oxylipins separation. The mobile phase A was water with 0.1% acetic acid while the mobile phase B was composed of acetonitrile/methanol (80/15, v/v) and 0.1% acetic acid. Gradient elution was performed at a flow rate of 250 μL/min and the gradient used is described in the attached table. The injection volume was 10 μL and the samples were kept at 4°C in the auto sampler. Analytes were detected by negative MRM mode using a 4000 QTrap tandem mass spectrometer (Applied Biosystems Instrument Corporation, Foster City, CA) equipped with an electrospray ionization source (Turbo V). The QTrap was set as follows: CUR = 20 psi, TEM = 500°C, GS1 = 50 psi, GS2 = 30 psi, CAD = High, IS = −4500 V, DP = −60 V, EP = −10 V. Calibration curves were generated by 10 μL injections of seven standards containing each analyte, internal standard I, and internal standard II for quantification purpose.

Statistical Analysis

Each analyzed parameter was expressed as mean ± SD, unless otherwise stated, with at least three independent measurements. Continuous variables were compared with the Student’s t-test, whereas categorical variables were compared with Chi-square test. All statistical tests were two-sided, statistical significance was taken as P < 0.05.

RESULT

Effect of sEH Gene Deficiency on IBD-Induced Carcinogenesis in the Bowel

All of the mice including wild-type, sEH(−/−), IL-10(−/−), and sEH(−/−)/IL-10(−/−) mice were monitored daily. In order to synchronize the development of IBD, the mice were fed an AIN 93M diet containing 200 ppm of piroxicam for 1 wk (at age 4 wk) [28]. Early mucosal injury in the bowel was observed 10 d after piroxicam use; IL-10(−/−) mice, sEH(−/−)/IL-10(−/−) mice, sEH(−/−) mice, and wild-type control animals all exhibited mild mucosal ulcer formation in the cecum and proximal small bowel. sEH(−/−)/IL-10(−/−) and sEH(−/−) mice showed much milder ulcerations as compared to the IL-10(−/−) mice. While the sEH(−/−) and wild-type control mice showed a full recovery from the erosion and ulceration 4 wk after piroxicam use, the sEH(−/−)/IL-10(−/−) and IL-10(−/−) mice continued to display chronic active IBD (n = 3 mice each strain each gender mice). Thus, the IBD process was synchronously induced in the sEH(−/−)/IL-10(−/−) and IL-10(−/−) mice at 4 wk postpiroxicam treatment.

At age 17 wk, male IL-10(−/−) and sEH(−/−)/IL-10(−/−) mice started to display loss of body weight and progression worsened. At age 25 wk, more than 50% male IL-10(−/−) mice and sEH(−/−)/IL-10(−/−) mice showed >20% decrease of body weight as compared to male wild-type mice. Male IL-10(−/−) mice displayed more severe diarrhea and bowel inflammation than male sEH(−/−)/IL-10(−/−) mice. But male sEH(−/−)/IL-10(−/−) mice exhibited smaller prostate and testicles, indicating deduction of body weight probably due to alteration of testosterone [29]. Female sEH(−/−)/IL-10(−/−) mice did not exhibited any gross abnormalities until age 38 wk.

Extensive pathological analysis was performed for the entire gastrointestinal tract of all mice. In wild-type and sEH(−/−) mice, no tumor or inflammation was observed. In IL-10(−/−) mice, 85% mice (17/20) developed polypoid tumor masses (Figure 1A), 53% tumors located in duodenal/proximal small bowel and 47% in cecum/rectal colon with average 2 ± 0.9 tumors/mouse (tumor multiplicity) and average tumor volume 344.55 ± 71.73 mm³. Histopathology revealed that most tumors were polypoid well to moderately differentiated adenocarcinoma with invasion into laminar propria or submucosa (Figure 1B), and only one mouse developed an invasive mucinous carcinoma (Figure 1C and D). Compared to IL-10(−/−) mice, a significant decrease of
tumor multiplicity and volume was observed in sEH(−/−)/IL-10(−/−) mice that had average 1 ± 0.3 tumor/mouse (Figure 1E) and average tumor volume 126 ± 23.18 mm³ (Figure 1F, P = 0.028). In sEH(−/−)/IL-10(−/−) mice, 33% tumors occurred in the duodenum/proximal small bowel and 67% in the cecum and right colon; but no statistical difference was observed for tumor location as well as for genders, as compared to IL-10(−/−) mice. Histopathology revealed that all of the intestinal tumors in sEH(−/−)/IL-10(−/−) mice were polypoid well differentiated adenocarcinoma, and no invasive mucinous carcinoma was seen.

IBD-induced dysplasia was analyzed histopathologically and further characterized with Ki-67-labeled proliferative cells. Morphologically, IBD-induced dysplasia was characterized as the partial loss of nuclear polarity and cell maturation with nuclear atypia, and an increase in mitotic figures, as seen in Figure 2A and B. Ki-67-labeled proliferative cells were observed in the crypt epithelium and extended to the luminal surface in the dysplastic lesion, as shown in Figure 2C and D. In nondysplastic mucosa, Ki-67-labeled proliferative cells were only observed in the crypt epithelium. As summarized in histogram in Figure 2E, 90% (18/20) of IL-10(−/−) mice showed a significant decrease of tumor multiplicity in sEH(−/−)/IL-10(−/−) mice (n = 20) as compared to IL-10(−/−) mice (n = 19, P = 0.01). (F) Distribution of tumor size: the tumor size in sEH(−/−)/IL-10(−/−) mice (n = 20) was significantly smaller than that in IL-10(−/−) mice (n = 19). Results were reported as mean ± SD. The statistical difference between sEH(−/−)/IL-10(−/−) and IL-10(−/−) animals was calculated and labeled in the figure.

Figure 1. Morphology of colitis-induced adenocarcinoma and histopathology of mucosal inflammation.

**Effect of sEH Gene Deficiency on Intestinal Chronic Active Inflammation**

Inflammatory activity in the intestinal tract was semi-quantitatively analyzed histopathologically including the parameters of inflammation-involved area, inflammatory cell infiltration, inflammation-caused reactive epithelial hyperplasia, and ulcer formation according to our established criteria [22]. In IL-10(−/−) mice, 90% (18/20) of IL-10(−/−) mice showed extensive and intense inflammation with ulcer formation in the bowel (Figure 3A), and the average inflammatory scores were 1.40 ± 0.18 for inflammation-involved area, 2.15 ± 0.18 inflammation cell infiltration, and 1.45 ± 0.18 for inflammation-caused reactive epithelial hyperplasia, furthermore 35% IL-10(−/−) mice had large active ulcer (Figure 3A) with extensive myeloperoxidase-labeled neutrophil infiltration (Figure 3C). Compared to IL-10(−/−) mice, only 45% (9/20) of sEH(−/−)/IL-10(−/−) mice showed focal mild inflammation (Figure 3B), and the average inflammatory scores were 1.15 ± 0.18 for inflammation-involved area, 2.00 ± 0.30 inflammation cell infiltration, and 1.05 ± 0.18 for inflammation-caused reactive epithelial hyperplasia, showing well-differentiated mucinous carcinoma invading into muscularis propria.
were 0.70 ± 0.16 for inflammation-involved area \( (P = 0.007) \), 1.55 ± 0.35 for inflammation cell infiltration \( (P = 0.032) \), and 0.95 ± 0.21 for inflammation-caused reactive epithelial hyperplasia \( (P = 0.044) \), as summarized in histogram in Figure 3E. Only 10% sEH\(-/-\)/IL-10\(-/-\) mice developed small active ulcers (10% vs. 35%, \( P < 0.05 \)). MPO immunohistochemical staining was performed...
to analyze active neutrophil infiltration in the inflamed areas. Compared to that of IL-10(−/−) mice (Figure 3C), a much significant decrease of MPO-positive neutrophils in the inflamed areas was observed in sEH(−/−)/IL-10(−/−) mice (Figure 3D). Overall inflammatory index (sum of each parameter) was significantly decreased in sEH(−/−)/IL-10(−/−) mice compared to that of IL-10(−/−) mice (3.3 ± 0.74 vs. 5.35 ± 0.46, P = 0.003, Figure 3E).
Analysis of Key Inflammatory Cytokines and Chemokines As Well As NF-κB and VCAM-1 Signals With Quantitative Real-Time-PCR and Western Blot Approaches

Transcriptional expressions of inflammatory cytokines and chemokines were analyzed quantitatively using real-time-PCR approach for freshly collected colonic mucosa (n = 6 mice/group). As seen in Figure 4, all of wild-type mice showed minimal expression of IFN-γ, TNF-α, MCP-1, IL-12, IL-17, and IL-23. By setting the value of each cytokine expression in wild-type mice as 1, a minimal increase of MCP-1, IL-17, and IL-23 was seen in sEH(C0/C0/C0), but did not reach a statistical significance. A significant increase of the mRNA expressions in these cytokines/chemokines was found in IL-10(C0/C0/C0) mice as compared to wild-type animals, including 13-fold increase in TNF-α, 5-fold increase in IFN-γ, 10-fold increase in MCP-1, 5-fold increase in IL-12, and 4-fold increase in both IL-17 and IL-23. Compared to IL-10(−/−) mice, a highly significant decrease of the mRNA expression in these inflammatory cytokines and chemokines was observed in sEH(−/−)/IL-10(−/−) mice and showed 7-fold decrease in TNF-α, 3-fold decrease in IFN-γ, 8-fold decrease in MCP-1, 4-fold decrease in IL-12, 1-fold decrease in IL-17, and 2-fold decrease in IL-23 as compared to IL-10(−/−) mice, as seen in Figure 4 (P < 0.01).

Expressions of total and phosphorylated NF-κB, as well IkκB and VCAM-1 were measured using the Western blotting approach and densitometry analysis for whole tissue/cell lysates of freshly collected colonic mucosa (n = 6/group). As shown in Figure 5A and B, all of these inflammatory signals were significantly decreased in sEH(−/−)/IL-10(−/−) mice as compared to those in IL-10(−/−) mice.

Plasma Levels of Eicosanoid Profile Analyzed With a Liquid Chromatography-Tandem Mass Spectrometry (LC/MS-MS) Assay

Quantitative analysis of eicosanoids profiles is far more significant than individual biomarker(s) for evaluating arachidonic acid metabolic pathways. Simply, the ratios of fatty acid epoxides (EpOMEs) to their corresponding diols (DiHOMEs) and EETs to DHETs are the most crucial biomarkers for determining sEH activity. With LC/MS-MS approach, we analyzed plasma levels of eicosanoid profile in these animals. Compared to wild-type mice, sEH(−/−) exhibited a significant increase of EETs, including 11(12)-, 14(15)-EET, and the fatty acid epoxides (EpOME), including 9(10)- and 12(13)-EpOME, as well as a significantly decrease of corresponding fatty acid diols, including 9(11)-DHOME and 12(13)-DHOME, and the DHETs, including 11(12)-DHET, and 14(15)-DHET, as seen in Figure 6. As expected, ratios of EETs to DHETs and EpOMEs to DiHOMEs were significantly increased in sEH(−/−) mice.

In IL-10(−/−) mice (IBD mice), a significant increase of fatty acid diols, including 9(11)-DHOME...
and 12(13)-DHOME, and the DHETs, including 11(12)-DHET, and 14(15)-DHET was observed as compared to wild-type mice (P < 0.05, Figure 6) but no change was seen in the levels of EETs (including 11(12)-, 14(15)-EET) and the fatty acid epoxides (EpOME) (including 9(10)- and 12(13)-EpOME), nor the ratios of EETs to DHETs and EpOMEs to DiHOMEs (Figure 6). Compared to IL-10(-/-) mice, sEH(-/-)/IL-10(-/-) mice displayed a significant elevation of the levels of EETs (including 11(12)-, 14(15)-EET) and fatty acid epoxides (EpOME, including 9(10)- and 12(13)-EpOME), and a significant decrease of the levels of corresponding fatty acid diols (including 9(11)-DHHOME and 12(13)-DHHOME) and DHETs (including 11(12)-DHET and 14(15)-DHET), as seen in Figure 6. sEH(-/-)/IL-10(-/-) mice exhibited a much more significant increase in the ratios of EETs to DHETs and EpOMEs to DiHOMEs as compared to IL-10(-/-) mice (Figure 6).

To investigate if there was an effect of sEH gene deficiency on modulating other pathways of arachidonic acid metabolism, the key cyclooxygenase (COX2) and lipoxygenase (LOX)-mediated metabolites were analyzed, including PGE2 (prostaglandin E2), PGD2 (prostaglandin D2), and TXB2 (thromboxane B2) as products of COX2 pathway, and LTb4 (leukotriene B4) and S-HETE (5-hydroxyeicosatetraenoic acid) as metabolites of LOX pathway. As shown in Figure 7, the level of PGE2 was significantly increased in IBD mice (both IL-10(-/-) and sEH(-/-)/IL-10(-/-) mice) as compared to wild-type and sEH(-/-) mice; but a slight decrease of PGE2 was seen in sEH(-/-)/IL-10(-/-) mice as compared to IL-10(-/-) mice (no statistically significant). The level of LTb4 was significantly increased in sEH knockout mouse (both sEH(-/-) mice and sEH(-/-)/IL-10(-/-) mice) as compared to wild-type and IL-10(-/-) mice (Figure 7). PGD2 was markedly increased in sEH(-/-)/IL-10(-/-) mice as compared to IL-10(-/-) mice (Figure 7, no statistically significant). TXB2 and 5-HETE was mildly increased in sEH knockout mouse (both sEH(-/-) mice and sEH(-/-)/IL-10(-/-) mice) as compared to wild-type and IL-10(-/-) mice (Figure 7, no statistically significant).

DISCUSSION

The studies have demonstrated the beneficial effects of sEH gene deficiency on protection against myocardial ischemia-reperfusion injury, the inflammatory response to cerebral ischemia, and on improvement of glucose homeostasis using a powerful sEH gene knockout model in mice [16-18]. In the present study, we have first reported that sEH gene deficiency in IL-10 knockout mice led to the reduction of IBD inflammatory activity and IBD-induced tumor development in the bowel.

Long-standing chronic active IBD is at high risk for cancer development. Several molecular events involved in chronic active inflammatory processes contribute to cancer development, including the overproduction of reactive oxygen and nitrogen species, aberrant metabolites of key arachidonic acid metabolism, overproduction of cytokines/chemokines, and dysfunction of immunity system [2]. Inflammatory cells (including neutrophils, macrophages, lymphocytes, and plasma cells, as well as eosinophils and mast cells) play a central role in these molecular events. Reduction of inflammatory cell infiltration is crucial for suppressing inflammatory activity and inhibiting inflammation-induced carcinogenesis. sEH gene deficiency in IL-10(-/-) mice resulted in a significant decrease of inflammatory cell infiltration (mainly including neutrophils and macrophages) in the inflamed areas of bowel which was correlated with the reduction of glucose homeostasis.
IBD-induced tumor development. A simple mechanism involved in inhibiting inflammatory cell infiltration in sEH(−/−)/IL-10(−/−) mice directly links to abolish the hydrolysis of EETs by sEH knockout that results in an increase of EETs and further leads to inhibit NF-κB and IKK signals, VCAM-1 expression, and inflammatory cell infiltration [10]. Our qPCR analysis further revealed a significant decrease of mRNA expression of several key cytokines and chemokines including TNF-α, MCP-1, IL-12, IL-17, and IL-23. Although the mechanism for inhibition of these cytokines and chemokines by sEH knockout is not known, these inflammatory mediators play an important role in IBD. Studies in clinical and experimental IBD have shown the upregulation of various chemokines including TNF-α and MCP-1 in the colonic mucosal tissue. There are a significant attenuation of colitis and reduction of mortality in TNF-α and MCP-1-deficient mice. IL-12 is a well-studied proinflammatory cytokine that drives the pathogenic CD4 T cells responding against the bacterial microflora [30]. IL-23 is an additional member of IL-12 family [31]. Studies in animal models of IBD have revealed that IL-23 plays a key role in chronic intestinal inflammation. Selective depletion of IL-23 using monoclonal antibodies specific for the p19 subunit or by genetic ablation of the p19 gene greatly attenuates T cell-dependent colitis in T cell transfer model in mice [32,33] and inhibits spontaneous IBD development in IL-10(−/−) mice [34]. IL-23 drives an increased production of IL-17 and interferon (IFN)-γ by both T cells and non-T cells. In IBD, the major action of IL-17 is to promote additional inflammatory cascades by stimulating the production of chemokines leading to activate granulocytes[35,36], and to promote development of IBD-associated colorectal cancer [37].
NF-κB has been proposed to be a main molecular link between inflammation and carcinogenesis. NF-κB is not only a key signal transmitting cytokine signal cascade to the targeted cells such as endothelial cells for recruiting inflammatory cells into inflamed tissues, but also involved in driving tumor invasion and metastasis, particularly via its downstream products of matrix metalloproteinases and serine proteases, as well as anti-apoptotic factors such as Bcl-2 and Bcl-XL, etc [38]. Thus, downregulation of NF-κB and IKK signals in IBD in sEH deficient mice may play a critical role in IBD and its induced tumor development [39].

In addition to stabilization of EETs by abolishing sEH function through its gene knockout, whether or not there is any effect on modulating other pathways in arachidonic acid metabolism is an important concern. Our result revealed that LOX-mediated pathway was mildly regulated by sEH gene knockout and showed a mild increase of LTB4 and 5-HETE in sEH(−/−) mice and sEH(−/−)/IL-10(−/−) mice. This finding may represent a feedback regulation of inflammatory metabolites of arachidonic acid metabolism. However, COX-mediated key product PGE2 appears no change between wild-type mice and sEH(−/−) mice. PGE2 production via COX-1 and COX-2 is a key event to promote inflammation-induced carcinogenesis [40,41] and PGE2 production via COX-1 promotes intestinal stem cell survival and proliferation [42]. Our finding showed that the high elevation of PGE2 in IBD in IL-10(−/−) mice was mildly decreased in IL-10(−/−) mice with sEH gene deficiency.

A recent report indicates that endothelium-derived EETs and its induced VEGF appear to promote angiogenesis and tumor metastasis [43], which is contradictory to our findings. However, several studies in the literatures and our present results indicate that targeting sEH may be more beneficial for preventing inflammation-induced carcinogenesis rather than promoting it. Evidence includes (1) at least one-third of human cancer are associated with chronic inflammatory process in which active inflammatory cells (neutrophils and macrophages) play a critical role in IBD and its induced tumor development [39].

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growth, angiogenesis and metastatic potential in preclinical models [45,46]. (5) In the present study, using IBD-induced carcinogenesis in mice, we have observed a significant reduction of IBD-induced tumor development and no metastasis observed through extensively histopathological analysis for all key organs in IL-10(−/−) mice with sEH deficiency.

In conclusion, our results imply that sEH plays an important role in IBD and its-induced carcinogenesis. Simply, an increase of EETs to DHETs ratio including the stabilization of EETs and reduction of DHETs, is a key function for sEH gene deficiency. These functional changes in sEH knockout mice lead to attenuation of IBD inflammatory activities and its induced carcinogenesis, mechanistically via downregulation of cytokines and chemokines, NF-κB signals and VCAM1. Our results may have brought up a new direction of investigation on preventive and therapeutic strategy for IBD via targeting sEH.

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