Activation of HIF-1α does not increase intestinal tumorigenesis

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INFLAMMATORY BOWEL DISEASE (IBD) is a chronic inflammatory disease of the intestine with an unclear etiological mechanism. Currently, the paradigm proposed is a feed-forward synergistic dysregulation of the mucosal epithelial barrier, the mucosal immune system, and gut microbiota (2, 6, 52). Metagenomic and genome-wide association studies have clearly confirmed the role of all three in the progression of IBD (12, 20, 24, 35, 39). Several proinflammatory mediators are activated by HIF-1α and HIF-2α. These highly homologous transcription factors are induced in hypoxic foci and regulate cell metabolism, angiogenesis, cell proliferation, and cell survival. HIF-1α and HIF-2α are activated early in cancer progression and are important in several aspects of tumor biology. HIF-1α and HIF-2α have overlapping and distinct functions. In the intestine, activation of HIF-2α increases inflammation and colon carcinogenesis in mouse models. Interestingly, in ischemic and inflammatory diseases of the intestine, activation of HIF-1α is beneficial and can reduce intestinal inflammation. HIF-1α is a critical transcription factor regulating epithelial barrier function following inflammation. The beneficial value of pharmacological agents that chronically activate HIF-1α is decreased due to the tumorigenic potential of HIFs. The present study tested the hypothesis that chronic activation of HIF-1α may enhance colon tumorigenesis. Two models of colon cancer were assessed, a sporadic and a colitis-associated colon cancer model. Activation of HIF-1α in intestinal epithelial cells does not increase carcinogenesis or progression of colon cancer. Together, the data provide proof of principle that pharmacological activation of HIF-1α could be a safe therapeutic strategy for inflammatory bowel disease.

hydroxylation (37). In a cellular environment with sufficient oxygen, PHDs hydroxylate HIF-1α and HIF-2α, leading to von Hippel-Lindau gene (VHL)-induced proteasomal degradation (21, 22). Knockout mouse models and chemical inhibitors of PHDs demonstrate a robust activation of HIF signaling and underscore the importance of PHDs in the regulation of HIFs (37).

HIF-1α and HIF-2α have distinct functions in intestinal inflammation. HIF-2α has been shown to increase inflammation through activation of epithelial inflammatory response (44, 53). Several proinflammatory mediators are activated by HIF-2α. Mice overexpressing HIF-2α specifically in the intestinal epithelial cells demonstrate spontaneous colitis and are significantly more susceptible to intestinal injury in several mouse models of colitis (53). However, HIF-1α in intestinal epithelial cells is a potent protective factor through regulation of a battery of genes important in maintaining the epithelial tight barrier and mucosal immune response (3, 13, 26, 28, 34, 39, 48).

Currently no known adherens or tight junction proteins have been shown to be direct targets of HIF-1α in the intestine. However, several direct target genes of HIF-1α are expressed on the apical surface of intestinal epithelial cells and are critical in maintaining barrier integrity. Disruption of intestinal HIF-1α clearly demonstrates the central role of HIF-1α-induced barrier function in IBD (26).

PHD inhibitors or PHD knockout mice, which potently activate HIF-1α, have been shown to be beneficial in several mouse models of colitis (8, 28, 39, 49). However, the tumor-promoting effect of HIF-1α activators poses a major concern and limitation for their therapeutic use in the treatment of IBD. The colon tumor microenvironment shares several similar characteristics as the inflammatory foci in IBD. HIF-1α and HIF-2α are robustly increased in cancer (29, 42). Chronic activation of HIF-2α in intestinal epithelial cells potentiates tumorigenesis in mouse models of colon cancer (54, 55). Currently it is not known whether activation of HIF-1α can also increase colon carcinogenesis in mouse models. In the present study, we tested the hypothesis that activation of HIF-1α in intestinal epithelial cells would increase colon carcinogenesis. A mouse model of constitutively expressed HIF-1α in intestinal epithelial cells was characterized by whole-genome mRNA expression analysis. Moreover, the activation of HIF-1α in colon cancer was assessed in a sporadic intestinal tumor model and a colitis-associated colon cancer model. HIF-1α significantly increased glycolytic genes. However, constitutive activation of HIF-1α did not significantly alter tumor multiplicity, size, proliferation, or apoptosis. The data demonstrate that increase in HIF-1α in intestinal epithelial cells does not result in spontaneous tumor formation or further potentiate the progression of colon cancer.

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MATERIALS AND METHODS

Animals and treatments. The Hif-1αLSL/LSL and littermate controls were previously described (53). The Adenomatous polyposis coli (Apc)min/+ were purchased from The Jackson Laboratory. To investigate the role of HIF-1α in colorectal cancer, Hif-1αLSL/LSL mice were crossed with Apcmin/+ mice to generate Hif-1αLSL/LSL/Apcmin/+ and Hif-1α+/+ /Apcmin/+ mice. All mice are on C57BL/6 background, maintained in standard cages in a light- and temperature-controlled room, and were allowed standard Chow and water ad libitum. For the azoxymethane (AOM)/dextran sulfate sodium (DSS) study, 6-wk-old Hif-1αLSL/LSL and Hif-1α+/+ mice were injected intraperitoneally with AOM (10 mg/kg body wt). It should be noted that no dysplasia was observed in mice receiving a single AOM dose. Seven days following AOM injection, the Hif-1αLSL/LSL mice and littermate controls received water with 1.5% DSS for 7 days (inflammatory phase). The mice were placed on regular drinking water for 14 days (recovery phase), and two more inflammatory and recovery cycles were performed. All animal studies were carried out in accordance with Institute of Laboratory Animal Resources guidelines and approved by the University Committee on the Use and Care of Animals at the University of Michigan (UCUCA approval number: 10299).

Mucosal cell isolation. The jejunum and proximal large intestine were flushed with 1× PBS and cut open longitudinally. Mucosal scraping was performed to enrich for epithelial cells using a glass slide. It should be noted that isolated mucosal cells contain stromal cells as well as intraepithelial lymphocytes. The cells were used for Western blot or gene expression analysis as detailed below.

Western blot analysis. For Western blot analysis, nuclear fractions were prepared from the epithelial cells of the small intestine and colon using a hypertonic buffer (200 mM sucrose, 10 Tris·HCl, and 2 mM EDTA with protease inhibitors). Nuclear proteins were then extracted using a hypertonic buffer (200 mM sucrose, 10 Tris·HCl, and 2 mM EDTA) and proteins were resolved on SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane and probed with specific antibodies against HIF-1α (Santa Cruz, CA) and histone H3 (Cell Signaling Technology, Danvers, MA).

Histology, immunohistochemistry, and immunofluorescence. Mice were killed following a 2-h treatment of 100 mg/kg of 5-bromo-2-deoxyuridine (BrdU) (Sigma, St Louis, MO). Paraffin-embedded slides were deparaffinized in xylene and rehydrated in an ethanol gradient. Immunohistochemical analysis was performed with SYBR green dye and run on a 7900HT Fast Real-Time PCR System. cDNA was quantified using RIPA buffer, and proteins were resolved on SDS-PAGE. Protein fractions were then transferred onto a nitrocellulose membrane and probed with specific antibodies against HIF-1α (Santa Cruz, CA) and histone H3 (Cell Signaling Technology, Danvers, MA).

Quantitative real-time RT-PCR. RNA was isolated and reverse transcribed as previously described (55). For the AOM/DSS-treated normal tissue and tumor tissue following isolation, the RNA was further purified by lithium chloride precipitation. cDNA was quantified using SYBR green dye and run on a 7900HT Fast Real-Time RT-PCR system (Life Technologies, Carlsbad, CA) (primers listed in Table 1). Ct values were normalized to β-actin and expressed as fold difference from controls.

cDNA microarray analysis. RNA was extracted from colon mucosal scrapings from 8-wk-old Hif-1αLSL/LSL and littermate control mice and reverse transcribed. cDNA was hybridized to the Mouse Gene 1.1 ST Array Strips (Affymetrix, Santa Clara, CA), and data were analyzed as previously described (55) and compared with gene expression analysis from the colons of intestine-specific Vhl knockout (VhlHIE) mice. The full data set is available on the GEO database accession number GSE55915 for the Hif-1αLSL/LSL mice and accession number GSE36091 for the VhlHIE mice.

Statistics. Results are expressed as means ± SD. P values were calculated by independent t-test, one-way ANOVA, Dunnett’s t-test, and two-way ANOVA. P < 0.05 was considered significant.

RESULTS

Activation of intestinal HIF-1α does not lead to gross histological changes in the small intestine and colon. To directly understand the role of HIF-1α in the intestinal epithelium, the Hif-1αLSL/LSL mouse model was assessed (53). These mice overexpress an oxygen-stable HIF-1α in intestinal epithelial cells (Fig. 1A). The Hif-1αLSL/LSL mice do not have any overt phenotypes compared with littermate controls (53). Histological analysis of the small intestine and the colon also demonstrated no obvious difference compared with wild-type mice (Fig. 1B).

Global gene expression analysis in the colons of Hif-1αLSL/LSL mice. To better characterize the changes in the Hif-1αLSL/LSL mice, whole-genome mRNA analysis was performed on scraped colonic mucosal cells from the Hif-1αLSL/LSL and age-matched wild-type littermates. The microarray analysis using a twofold cutoff demonstrated that 34 genes were up-regulated and 16 genes were downregulated in Hif-1αLSL/LSL mice compared with littermate controls (Tables 2 and 3). As expected, several well-characterized target genes were induced, including genes involved in glycolysis, autophagy, and mitochondrial metabolism. The microarray data were compared with whole-genome mRNA analysis obtained from colons of VhlHIE mice (55). Disruption of VHL led to a robust
activation of HIF-1α and HIF-2α signaling, and the Hif-1αLSL/LSL mice demonstrated significant overlap with the Vhlnull mice with respect to genes that are increased (Fig. 2A and Table 4). Interestingly, only one gene demonstrated overlap in genes that were downregulated from the Hif-1αLSL/LSL and Vhlnull mice (Fig. 2A and Table 4). qPCR was performed on known HIF-1α target genes for verification that HIF-1α signaling was activated in the Hif-1αLSL/LSL mice (Fig. 2B). Several known HIF-1α target genes were significantly induced (phosphoglycerate kinase 1, Pgk1; procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2, Plod2; pyruvate dehydrogenase kinase, isozyme 1, Pdk1; macrophage migration inhibitory factor, Mif; prolyl 4-hydroxylase, alpha polypeptide 1, P4ha1; egl nine family homolog 3, Egln3; carbonic anhydrase 9, Car9; stanniocalcin 2, Stc2; aldolase c, Aldoc; family with sequence similarity 162, member A, Fam162a; NADH dehydrogenase 1 alpha subcomplex, 4-like 2, Ndufa4l2; and BCL2/adenovirus E1B 19kDa interacting protein 3, Bnip3) (1, 4, 15, 16, 23, 30, 31, 33, 43, 45, 50, 51). Moreover, novel putative HIF-1α-dependent genes that may impact tumor metabolism (serine hydroxymethyltransferase 2; growth regulation by estrogen in breast cancer 1; neuritin 1; adenylate kinase 4; myosin, light polypeptide 7, regulatory; and solute carrier family 16 member 3) were also assessed by qPCR analysis to confirm the results of the microarray (Fig. 2B). Together, these data provide evidence that Hif-1αLSL/LSL mice are an appropriate model to assess the role of HIF-1α activation in colon cancer. Interestingly, prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2, COX-2) a HIF-1α target gene critical in the growth of colon cancer through the production of prostaglandin E2 (PGE2), was not induced in the Hif-1αLSL/LSL mice. Loading was assessed by histone H3.
mice (25). Similarly, PGE synthase (PGES1), the downstream gene of COX-1 important in the production of PGE2, was also not induced in the colon of Hif-1αLSL/LSL mice (Fig. 2B). These data further confirm a previous study demonstrating a critical and specific role of HIF-2α in the regulation of the COX-2-mPGES1-PGE2 axis in colon cancer (54).

**Activation of intestinal HIF-1α does not increase sporadic colon tumorigenesis.** Hif-1αLSL/LSL and littermate controls were crossed to the Apcmin/+ mice. Apcmin/+ mice have a truncated mutation at codon 850 of the murine Apc gene (47). APC mutations are observed in over 80% of patients with sporadic colon cancer (10). The Apcmin/+ mice develop mostly small intestinal adenomas (47). Consistent with these data, Hif-1αLSL/LSL/Apcmin/+ mice developed predominantly small intestinal tumors. However, no significant increase in small intestinal tumors were observed in Hif-1αLSL/LSL/Apcmin/+ compared with littermate control mice (Hif-1αLSL/LSL) (Fig. 3, A and B). Similarly, HIF-1α activation did not lead to an increase in colon tumors in the Apcmin/+ mice (Fig. 3, A and C). Also the tumors in the small intestine and colon were all adenomas, and no increase in progression was observed in Hif-1αLSL/LSL/Apcmin/+ mice compared with littermate controls (Fig. 3A). The tumors were further analyzed for proliferation and apoptosis by BrdU and cCASP3 staining, respectively. However, no significant difference in tumor proliferation or apoptosis was observed in the Hif-1αLSL/LSL/Apcmin/+ compared with the littermate control mice (Fig. 4).

**Activation of intestinal HIF-1α does not increase colitis-associated colon cancer.** The Apcmin/+ mice are not a robust model for colon cancer, and therefore a colitis-associated colon cancer (CAC) mouse model was assessed (5). Body weight is a good index of systemic inflammation following DSS, and the littermate control mice had decreased body weight throughout the inflammatory and recovery phase compared with Hif-1αLSL/LSL mice (Fig. 5A). These results demonstrated that activation of intestinal HIF-1α significantly protected from...
DSS-induced injury compared with wild-type littermates. However, repeated exposures to DSS led to no significant difference in local inflammation in Hif-1α/H9251 LSL/LSL and wild-type mice, as assessed by colon length and histology (Fig. 5, B and C). The expression of cytokines that are critical in the progression of colon cancer such as TNF-α, IL-1β, and IL-6 were assessed by qPCR (18, 38). There was a significant decrease in TNF-α, IL-1β, and IL-6 in normal tissue from Hif-1α/H9251 LSL/LSL mice compared with wild-type mice. In the tumors, there was a significant decrease in TNF-α, but not IL-1β and IL-6 (Fig. 5D). Moreover, HIF-1α-dependent glycolytic and mitochondrial respiration genes important in cancer were significantly increased in tumor and normal tissue from Hif-1α LSL/LSL mice compared with wild-type mice. In the tumors, there was a significant decrease in TNF-α, but not IL-1β and IL-6 (Fig. 5D). Moreover, HIF-1α-dependent glycolytic and mitochondrial respiration genes important in cancer were significantly increased in tumor and normal tissue from Hif-1α LSL/LSL mice compared with wild-type mice. In the tumors, there was a significant decrease in TNF-α, but not IL-1β and IL-6 (Fig. 5D). Moreover, HIF-1α-dependent glycolytic and mitochondrial respiration genes important in cancer were significantly increased in tumor and normal tissue from Hif-1α LSL/LSL mice compared with wild-type mice. In the tumors, there was a significant decrease in TNF-α, but not IL-1β and IL-6 (Fig. 5D).

DISCUSSION

HIF-1α and HIF-2α are the two major transcription factors critical for hypoxic-mediated transcriptional and translational cellular response. In the intestine, HIF-2α has recently been shown to increase inflammation and colon cancer (53–55). HIF-2α can directly regulate several proinflammatory genes. TNF-α is a critical HIF-2α target gene in hypoxic inflammation (53). HIF-1α activation in the intestine leads to barrier protection and a decrease in several proinflammatory cytokines in mouse models of colitis (26, 28). However, the role of HIF-1α in cancer has led to concerns for the therapeutic use and efficacy of HIF-1α activators in IBD. The present data clearly demonstrate that, in mouse models of colon cancer, chronic activation of HIF-1α does not increase sporadic or inflammation-induced colon cancer. HIF-1α through a battery of target genes maintains barrier homeostasis and decreases inflammation in an acute colitis models (13, 26, 28, 34, 48). Consistent with these data, a significant decrease in proinflammatory mediators and a protection in body weight loss following DSS treatment in Hif-1α LSL/LSL mice compared with littermates control mice were observed. Tumor-elicited inflammation in sporadic colon cancer and inflammation in CAC are due to dysregulation of the intestinal epithelial barrier and activation of proinflammatory response (19). PHDs are critical in the activation of basal NF-κB and also in limiting IL-1β-induced NF-κB (11, 40). PHD inhibitors repress NF-κB activity, which is a critical signaling pathway in the progression of sporadic and colitis-associated colon cancer (17). PHD inhibitors may decrease tumor inflammation through two mechanisms, barrier protection and inhibition of NF-κB signaling, and therefore would be beneficial in patients with colon cancer. HIF-1α has been shown to have a major role in many aspects of tumor biology. A key function of hypoxia signaling in
Apc-driven tumors are shifting the tumor metabolism to anaerobic glycolysis. HIF-1α is a direct regulator of glycolytic genes, which we confirm in vivo in the present study. Moreover, Ndufa4l2 is robustly increased in the colon of Hif-1αLSL/LSL mice, and this gene has been shown to be critical in limiting oxygen consumption and cellular adaptation of cells to hypoxia (41, 50). The data demonstrate that the increase in these genes in normal colonic epithelial cells does not lead to cancer. Moreover, the increase in glycolytic and mitochondrial metabolic genes in adenomas does not increase the progression of early-stage tumors. Although most mouse models of colon cancer do not progress further than adenomas, it is possible that, in later stages of colon cancer, chronic activation of HIF-1α may potentiate progression. The microarray data from the Hif-1αLSL/LSL mice also demonstrated a significant increase in several novel genes that will require further experiments to confirm mechanisms of regulation by HIF-1α. Several downregulated genes were also identified. However, unlike the genes that were induced, there was not a good correlation between the downregulated genes in the Hif-1αLSL/LSL mice and the VhlΔHIE mice. This may be due to the role of HIF-1α as a transcription factor that can directly bind to promoters and activate gene expression, whereas, to date, no known direct repressed HIF-1α target genes have been confirmed. Interestingly, fatty acid binding protein-1 (Fapb1) is highly expressed in the intestine, and deletion results in significant protection in adenoma formation in the Apcmin/+ mice on 10% fat-containing diet (9). Although no protection was observed in adenoma formation in the Hif-1αLSL/LSL mice on standard chow, these data suggest that activation of HIF-1α may be beneficial in obesity-induced colon cancer.

The data provide strong evidence for the safe use of HIF-1α activators in colitis, without enhancing tumorigenesis. PHD inhibitors have been shown to be beneficial in mouse models of colitis and can activate the HIF response (8, 28, 39). In cancer-derived cell lines, PHD inhibitors can activate both HIF-1α and HIF-2α. In vivo in the intestine, PHD inhibitors primarily activate HIF-1α expression, thus limiting the proinflammatory role of HIF-2α (53). Moreover, three PHDs exist, PHD 1, 2, and 3 (also referred to as Egln-2, -1, and -3, respectively). The most commonly used inhibitors are pan antagonists of PHDs (37). However, through cell-based studies, it has been demonstrated that PHDs have different selectivity for HIF-1α and HIF-2α (37). Thus the pharmacological intervention to treat colitis using PHD inhibitors that can specifically activate either HIF-1α or HIF-2α response is feasible. Indeed, AKB-4924 is a HIF-1α-predominant PHD inhibitor, which is protective in mouse models of colitis (28).

In summary, the data demonstrate that in vivo activation of intestinal HIF-1α does not impact tumorigenesis in two mouse models of colon cancer. This provides evidence for the safe use of PHD inhibitors in the treatment of ischemic and inflammatory disorders of the intestine.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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