Id1 Deficiency Protects against Tumor Formation in \( \text{Apc}^{\text{Min/}} \) Mice but Not in a Mouse Model of Colitis-Associated Colon Cancer

Ning Zhang, Kotha Subbaramaiah, Rhonda K. Yantiss, Xi Kathy Zhou, Yvette Chin, Robert Benezra, and Andrew J. Dannenberg

Abstract

Different mechanisms contribute to the development of sporadic, hereditary and colitis-associated colorectal cancer. Inhibitor of DNA binding/differentiation (Id) proteins act as dominant-negative antagonists of basic helix–loop–helix transcription factors. Id1 is a promising target for cancer therapy, but little is known about its role in the development of colon cancer. We used immunohistochemistry to demonstrate that Id1 is overexpressed in human colorectal adenomas and carcinomas, whether sporadic or syndromic. Furthermore, elevated Id1 levels were found in dysplasia and colon cancer arising in patients with inflammatory bowel disease. Because levels of PGE\(_2\) are also elevated in both colitis and colorectal neoplasia, we determined whether PGE\(_2\) could induce Id1. PGE\(_2\) via EP4 stimulated protein kinase A activity resulting in enhanced pCREB-mediated Id1 transcription in human colonocytes. To determine the role of Id1 in carcinogenesis, two mouse models were used. Consistent with the findings in humans, Id1 was overexpressed in tumors arising in both \( \text{Apc}^{\text{Min/}} \) mice, a model of familial adenomatous polyposis, and in experimental colitis-associated colorectal neoplasia. Id1 deficiency led to a significant decrease in the number of intestinal tumors in \( \text{Apc}^{\text{Min/}} \) mice and prolonged survival. In contrast, Id1 deficiency did not affect the number or size of tumors in the model of colitis-associated colorectal neoplasia, likely due to exacerbation of colitis associated with Id1 loss. Collectively, these results suggest that Id1 plays a role in gastrointestinal carcinogenesis. Our findings also highlight the need for different strategies to reduce the risk of colitis-associated colorectal cancer compared with sporadic or hereditary colorectal cancer. Cancer Prev Res, 8(4), 303–11. ©2015 AACR.

Introduction

The pathogenesis of cancer varies depending on the underlying risk factors. Different mechanisms contribute to the development of hereditary, sporadic and colitis-associated colorectal cancer (1). The order of mutations in the multistep process of colon carcinogenesis differs, depending on the underlying etiology (2). In colitis-associated cancers, continuous tissue destruction and renewal along with oxidative damage can trigger mutagenesis and cancer initiation (2, 3). Given these fundamental differences, it seems likely that different approaches will be needed to reduce the risk of gastrointestinal neoplasia depending on whether or not chronic inflammation is involved. Little has been done to compare the impact of targeting specific molecules in models of hereditary versus inflammation-related gastrointestinal neoplasia.

The inhibitor of DNA binding/differentiation (Id) family includes four members (Id1–4), all of which lack a DNA-binding domain and act as dominant-negative antagonists of basic helix–loop–helix transcription factors, primarily E proteins (4–7). In a variety of cell contexts, Id proteins have been shown to regulate normal cell fate determination, proliferation, and differentiation (6). Id1 is overexpressed in numerous malignancies, including colorectal cancer and modulates tumor cell behavior (7–9). Forced overexpression of Id1 in transgenic mice has been associated with the development of small intestinal adenomas, although the effects in this study were non–cell autonomous (10). Overexpression of Id1 in cell lines can increase proliferative and metastatic potential (11). Id expression is also essential for the formation of patent tumor vasculature likely through its ability to enhance the mobilization of endothelial progenitor cells (12). For these reasons, Id1 is regarded as a potential target for cancer therapy (5). Whether Id1 deficiency will reduce the incidence of tumor formation in the gastrointestinal tract is uncertain.

Id1 can be induced by both oncogenes and inflammatory mediators (13). Several pro-proliferative/oncogenic stimuli, including c-Myc, Src, AML1-ETO, Kras, and VEGF, induce Id levels (14, 15). Proinflammatory stimuli can induce Id1 (13). COX-derived prostaglandin E\(_2\) (PGE\(_2\)), a bioactive lipid that plays a role in both inflammation and colon carcinogenesis, has been reported to induce Id1 in breast cancer cells (16). Importantly, we recently showed that loss of Id1 in colon stem cells sensitized mice to worse chemically induced colitis (17).

These observations suggest that the role of Id1 in carcinogenesis might vary depending on the nature of the initiating event. More specifically, oncogenic stimuli might require Id1 for optimal...
proliferative potential, but inflammation-induced neoplasia might be exacerbated by Id1 loss. In this study, we had three objectives. The first goal was to determine whether Id1 was overexpressed in human colorectal adenomas and cancers, including colitis-associated colorectal cancer. The second objective was to elucidate the mechanism by which PGE2 induces Id1 in colonocytes. Finally, we compared the effects of Id1 deficiency on gastrointestinal carcinogenesis in both Apc<sup>Min</sup> mice, a model of familial adenomatous polyposis (FAP), and a model of colitis-associated colorectal neoplasia. Here, we demonstrate that Id1, a PGE2-inducible gene, is overexpressed in both murine and human gastrointestinal neoplasia. Id1 deficiency protected against tumor formation in Apc<sup>Min</sup> mice, but not in a model of colitis-associated colorectal neoplasia.

Materials and Methods

Materials

Media to grow cells and Lipofectamine were from Invitrogen. Nitrocellulose membranes were from Schleicher & Schuell. Reagents for the luciferase assay were from Analytical Luminescence. Anti-Id1 antiserum was from BioCheck. Antiserum to pCREB was from Cell Signaling Technology. Western blotting objectives. The fl

Zhang et al.

Genosys. Chromatin immunoprecipitation (ChIP) assay kits were recommended. DNA

published online January 26, 2015; DOI: 10.1158/1940-6207.CAPR-14-0411

Published Online First January 26, 2015; DOI: 10.1158/1940-6207.CAPR-14-0411

transfections

Id1 promoter deletion and mutant constructs have been described previously (19). Cells were seeded at a density of 5 × 10⁴ cells/well in 6-well dishes and grown to 50% to 60% confluence. For each well, 2 μg of plasmid DNA was introduced into cells using 8 μg of Lipofectamine as per the manufacturer’s instructions. After 7 hours of incubation, the medium was replaced with basal medium. The activities of luciferase and β-galactosidase were measured in cellular extract.

Chromatin immunoprecipitation assay

ChIP assays were performed with a kit (Upstate Biotechnology) according to the manufacturer’s instructions. A total of 1 × 10⁶ cells were cross-linked in a 1% formaldehyde solution for 10 minutes at 37°C. Cells were then lysed in 200 μl of SDS buffer and sonicated to generate 200 to 1,000 bp DNA fragments. After centrifugation, the cleared supernatant was diluted 10-fold with ChIP buffer and incubated with 1.5 μg of the indicated antibody at 4°C. Immune complexes were precipitated, washed, and eluted as recommended. DNA–protein cross-links were reversed by heating at 65°C for 4 hours, and the DNA fragments were purified and dissolved in 50 μl of water. Each sample of 10 μl was used as a template for PCR amplification. The forward and reverse primers used for amplifying the Id1 promoter were 5’-AGCCGG-TCGGGTITTTACGTCC-3’ and 5’-CTTGTGTCGTGTCGTGTC-3’. This primer set encompasses the Id1 promoter segment, which includes the cAMP-response element (CRE). PCR was performed at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds for 30 cycles. The PCR products generated from the ChIP template were sequenced, and the identity of the Id1 promoter was confirmed.

Human tissues

We obtained formalin-fixed, paraffin-embedded tissue samples from normal human colon, as well as colorectal epithelial neoplasms that develop in several circumstances. These included colorectal adenomas that developed sporadically and in association with FAP, sporadic and syndromic colorectal cancers, and colitis-associated dysplasias and carcinomas. The Institutional Review Board at Weill Cornell Medical College approved this study.

Mouse models

Id1<sup>−/−</sup> mice on a C57BL6/j background were created in our laboratory (20). Apc<sup>Min</sup> mice were purchased from The Jackson Laboratory. Apc<sup>Min</sup> and Id1<sup>−/−</sup> mice were crossed to generate Apc<sup>Min</sup> Id1<sup>−/−</sup> mice. These mice were then crossed to produce Apc<sup>Min</sup> Id1<sup>+/−</sup>, Apc<sup>Min</sup> Id1<sup>+/+</sup>, and Apc<sup>Min</sup> Id1<sup>−/−</sup> mice. Genotypes were determined by PCR analysis of tail-tip–derived genomic DNA as previously described (21). All mice had a C57BL6/j genetic background and were allowed access to PicoLab irradiated rodent diet 20 (LabDiet) ad libitum. Mice were euthanized at 110 days of age. After flushing with PBS, the intestinal tract was divided into proximal and distal small intestine. Each segment was opened longitudinally and laid out on filter paper. The samples were fixed in 4% paraformaldehyde overnight at 4°C. In a separate experiment to evaluate the effects of Id1 status on survival, Apc<sup>Min</sup> Id1<sup>+/−</sup> and Apc<sup>Min</sup> Id1<sup>−/−</sup> mice were followed over time.

For the colitis-associated colorectal neoplasia model, 8-to10-week-old C57BL6/J Id1<sup>+/+</sup> and C57BL6/J Id1<sup>−/−</sup> male
mice were given a single i.p. injection of 12.5 mg/kg azoxymethane in 0.9% NaCl. Five days later, mice were treated with 2% DSS. DSS was given in drinking water for 5 days followed by regular water for 16 days. Two additional cycles of DSS treatment followed. During the third cycle, the concentration of DSS was reduced from 2% to 1%, and the duration of treatment was reduced from 5 to 4 days in an effort to reduce mortality. Ten days after completing the final DSS treatment, mice were sacrificed and the colon was removed and flushed with PBS. Longitudinally opened colon was then fixed in 4% paraformaldehyde overnight at 4°C. All experiments were approved by the Institutional Animal Care and Use Committee at Memorial Sloan Kettering Cancer Center.

Tumor measurements
After fixation, tissues were Swiss-rolled, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E). Histologic evaluation was carried out by a pathologist specializing in gastroenterology who was blinded to sample identity. Tumor numbers were quantified as number per H&E section. Tumor size was measured with a ruler under the microscope.

Immunohistochemistry
Tissue sections were blocked with 10% normal goat serum and 2% BSA for 30 minutes. Primary antibody incubations were done for 2 hours with anti–mouse-specific Id1 rabbit monoclonal antibody (BioCheck) or anti–mouse-human cross-specific Id1 rabbit monoclonal antibody followed by incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 8 minutes (22). A similar approach was used with the anti-myeloperoxidase rabbit antibody (Dako) at a dilution of 1:2,500. The endogenous alkaline phosphatase, and the 3,3’-diaminobenzidine detection kit were used according to the manufacturer’s instructions (Ventanna Medical Systems).

Statistical analysis
The nonparametric Wilcoxon rank-sum test and Kruskal-Wallis test were used to compare tumor numbers between and across experimental groups, respectively. Difference in the proportion of mice that developed tumors between or across experimental groups was evaluated using the Fisher exact test. Difference in proportion of tumors in a size category among mice in different experimental groups was examined using the quasi-binomial model. The log-rank test was used to compare mouse survival between experimental groups. All statistical tests were two-sided. Difference with a P value of <0.05 is
Figure 1. Id1 is expressed in human colorectal neoplasia. A, sporadic colonic adenomas contain straight tubules lined by dysplastic epithelial cells with cigar-shaped nuclei (top) compared with normal entrapped crypts (arrow) with abundant cytoplasm and small nuclei. B, adenomatous epithelium shows strong nuclear Id1 staining (top) compared with nonadenomatous crypts that express much lower levels of Id1 (arrow). Endothelial cells in the lamina propria also stain for Id1 (arrowhead). C, a colon cancer is composed of fused glands that contain malignant epithelial cells. D, Id1 positivity is present in tumor cells and the endothelium of vascular channels within the stroma (arrowhead, see inset). This tumor was from a patient with FAP. E, ulcerative colitis-associated low-grade dysplasia (arrowhead) is sharply demarcated from normal mucinous crypts (arrow). F, the dysplastic foci are strongly Id1 positive (arrowhead) compared with normal crypts that are negative (arrow). Id1-positive endothelial cells are present in the lamina propria between dysplastic crypts (inset).
PGE2 also induced Id1 mRNA in these cell lines (data not shown). Transient transfections were carried out to explore the mechanism by which PGE2 regulated Id1 transcription. As shown in Fig. 3B, PGE2 stimulated Id1 promoter activity. The Id1 promoter contains multiple regulatory elements (16, 19). Interestingly, mutating the CRE but not the Ebox or Egr-1 site abrogated the inductive effects of PGE2. Next, ChIP assays were performed to determine whether treatment with PGE2 stimulated the binding of pCREB to the Id1 promoter. Increased recruitment of pCREB to the Id1 promoter was observed following PGE2 treatment (Fig. 3C). Additional studies were carried out to explore the signal transduction pathway responsible for PGE2-mediated induction of Id1 transcription. PGE2 exerts its effects by binding to G protein–coupled receptors. Four subtypes of PGE2 receptor (EP1-4) can mediate the effects of PGE2. EP4 has been suggested to play a role in mediating the procarcinogenic effects of PGE2 (25). ONO AE3-208, an EP4 receptor antagonist, blocked PGE2-mediated induction of Id1 (Fig. 3D). Taken together, these data suggest that PGE2 binds to EP4 and thereby stimulates PKA activity leading to pCREB-dependent activation of Id1 transcription.

Id1 has different effects on tumor formation in Apc<sup>Min/−</sup> mice versus mice with colitis-associated colorectal neoplasia

To examine whether Id1 plays a role in intestinal tumorigenesis, Apc<sup>Min/−</sup> mice were interbred with Id1-deficient mice to generate mice of three genotypes: Apc<sup>Min/−</sup>/Id1<sup>+/+</sup>, Apc<sup>Min/−</sup>/Id1<sup>−/−</sup>, and Apc<sup>Min/−</sup>/Id1<sup>−/−</sup>. Tumor multiplicity was reduced by knocking out either one or both alleles of Id1 (Fig. 4A). Haploinsufficiency or partial reduction of Id1 protein levels has been shown in other systems to impact tumor progression (21, 26, 27). In contrast with tumor number, Id1 deficiency was not associated with a reduction in tumor size (Fig. 4B). Because loss of Id1
protected against tumor formation, a separate experiment was carried out to determine the effects of Id1 on the survival of Apc\textsuperscript{Min} mice. Consistent with the reduction in tumor burden, the lifespan of Apc\textsuperscript{Min} Id1\textsuperscript{+/−} mice was increased compared with Apc\textsuperscript{Min} Id1\textsuperscript{+/+} mice (Fig. 4C, P = 0.04).

To determine whether Id1 plays a role in colitis-associated tumor formation, wild-type (n = 20) and Id1\textsuperscript{−/−} (n = 19) mice were treated with azoxymethane followed by three cycles of DSS treatment to induce injury (Fig. 5A). Approximately 2 months after azoxymethane injection, Id1\textsuperscript{−/−} and wild-type controls were sacrificed and tumor burden was quantified. Tumor number and size were similar regardless of Id1 status (Fig. 5B and C).

**Discussion**

Id1 plays an important role in the regulation of lineage commitment and cell-cycle progression (4). It is not surprising, therefore, that high levels of Id1 are detected in many tumor types (7). Our data show that in contrast with the low and limited expression pattern of Id1 found in normal colonic crypts (17), Id1 is widely and strongly expressed in sporadic colorectal adenomas, adenomas arising in patients with FAP and in sporadic colorectal cancers. Id1 levels were also increased in both dysplasia and colorectal cancer arising in patients with inflammatory bowel disease. The fact that elevated levels of Id1 occur in colorectal adenomas and in dysplasia implies that upregulation of Id1 occurs as a relatively early event during tumorigenesis. Similar to the findings in humans, Id1 was overexpressed in both intestinal adenomas that arose in Apc\textsuperscript{Min} mice and in a mouse model of colitis-associated colorectal neoplasia.

In addition to Id1, levels of PGE\textsubscript{2} are increased in premalignant adenomas, colorectal cancer, and colitis (23, 24, 28). Previously, we reported that PGE\textsubscript{2} induced Id1 in breast cancer cells (16). Here, we demonstrated that PGE\textsubscript{2} induced Id1 in both primary human colonocytes and colon cancer cell lines. Interestingly, this increase reflected enhanced Id1 transcription. The inductive effect of PGE\textsubscript{2} was blocked by both an EP4 receptor antagonist and an inhibitor of PKA. Transient transfections and ChIP assays suggested the involvement of pCREB and the CRE site in the Id1 promoter. A similar response has been reported in breast cancer cells (16), where the PGE\textsubscript{2} antagonist ONO-AE3-208 blocked the induction of Id1.

Id1 is a transcription factor that is involved in the regulation of lineage commitment and cell-cycle progression. Our data show that high levels of Id1 are detected in sporadic colorectal adenomas, colorectal cancer arising in patients with inflammatory bowel disease, and in tumors arising in patients with FAP. The induction of Id1 by PGE\textsubscript{2} suggests that this factor may play a role in the development of these tumors. Further studies are needed to determine the exact role of Id1 in the development of colorectal cancer.
on tumor formation. Recently, we reported that Id1 deficiency in the colonic epithelium, including stem cells, sensitized mice to worse DSS-induced colitis and increased mortality (17). As mentioned above, colitis stimulates tumor development. Here, we compared the effects of Id1 deficiency in mouse models of sporadic and colitis-associated colorectal cancer. In theory, this comparison allowed us to determine whether the exacerbation of colitis induced by Id1 loss (17) would counter the antitumor activity related to Id1 loss. Although overexpression of Id1 is seen in both models, Id1 deficiency protected against tumor formation in the Apc\(^{min}+/+\) mouse, but not in the AOM/DSS model. The observed reduction in tumorigenesis in the Apc\(^{min}+/+\) model is not surprising, based on the numerous pro-tumorigenic effects of Id1 (5). On the basis of our recent discovery that Id1 is a functionally important stem cell marker (17), it is appealing to speculate that loss of Id1 either reduced the number or altered the function of cancer initiating cells leading to a decreased tumor burden. Indeed, O’Brien and colleagues (29) have shown recently that loss of Id1 and Id3 reduce the self-renewal capacity of colon cancer stem/initiating cells as a result of a decrease in p21 and more rapid stem cell exhaustion in primary colon cancer cell culture and xenograft analyses. With respect to the colitis-associated cancer model, the beneficial effect of Id1 deficiency on tumorigenesis was likely countered by the cancer-promoting effect of inflammation (30). In support of this possibility, numerous mechanisms have been identified that help explain the link between inflammation and colorectal cancer. Inflammatory cells produce reactive oxygen species, which can induce DNA damage and mutations (31). DNA methyl transferases can be induced during inflammation and may mediate the silencing of numerous genes involved in the pathogenesis of colorectal cancer (32). Activation of NF-kB, a common occurrence in inflammation, supports tumorigenesis by increasing cell proliferation and angiogenesis, and suppressing apoptosis (33). The different consequences of ablating Id1 in the two models are strikingly reminiscent of what was observed in Cox-2− deficient mice (34). Similar numbers of tumors were observed in Cox-2 deficient compared with wild-type mice in the AOM/DSS model, but reduced tumor numbers occurred in the Apc\(^{min}+/+\) model (34, 35). Cox-2 deficiency like loss of Id1 is associated with worse colitis (36). It is tempting to speculate that the effects of Cox-2 deficiency might be mediated by downregulation of Id1, given the similarity of the phenotypes and the direct link between Cox-2 expression, PGE\(_2\) production, and Id1 transcription. We note that, although the reduction in tumor numbers in Apc\(^{min}+/+\) mice upon Id1 deletion occur primarily in the small intestine, this result is of potential clinical significance as between 25% and 87% of patients with FAP have small intestinal tumors at the time of colectomy (37). Id1 antagonists are being developed and could prove useful in the management of small intestinal adenomas (38). Collectively, this study provides new insights into the role of Id1 in gastrointestinal carcinogenesis. Importantly, our results also strongly suggest that different chemopreventive strategies will be needed to reduce the risk of colitis-associated colorectal cancer compared with sporadic or hereditary colorectal cancer.

Figure 4.

Id1 deficiency suppresses small intestinal tumor multiplicity and increases survival in Apc\(^{min}+/+\) mice. A, in comparison with Apc\(^{min}+/+\) Id1−/− mice (n = 25), Apc\(^{min}+/+\) Id1−/− mice (n = 11) and Apc\(^{min}+/+\) Id1+/− mice (n = 11) developed reduced numbers of small intestinal tumors (P = 0.006 and 0.025, respectively). B, among mice that developed tumors, the proportions of tumors in different size categories were not statistically different among the three groups. C, survival of Apc\(^{min}+/+\) Id1−/− (n = 17) and Apc\(^{min}+/+\) Id1+/− (n = 19) mice was assessed. The log-rank test showed statistically significantly lower hazard of death in Apc\(^{min}+/+\) Id1−/− compared with Apc\(^{min}+/+\) Id1+/− mice (P = 0.04).
Disclosure of Potential Conflicts of Interest

R. Benezra has ownership interest (including patents) and is a consultant/advisory board member for Angiogenex (AGGX). No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: N. Zhang, R.K. Yantiss, R. Benezra, A.J. Dannenberg
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Zhang, K. Subbaramaiah, Y. Chin, A.J. Dannenberg
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X.K. Zhou, R. Benezra, A.J. Dannenberg
Writing, review, and/or revision of the manuscript: N. Zhang, K. Subbaramaiah, R.K. Yantiss, X.K. Zhou, R. Benezra, A.J. Dannenberg
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Chin, A.J. Dannenberg
Study supervision: A.J. Dannenberg

References

15. Sakurai D, Tsuchiya N, Yamaguchi A, Okajii Y, Tsuno NH, Kobata T, et al. Crucial role of inhibitor of DNA binding/differentiation in the vascular...


Id1 Deficiency Protects against Tumor Formation in $Apc^{Min/+}$ Mice but Not in a Mouse Model of Colitis-Associated Colon Cancer

Ning Zhang, Kotha Subbaramaiah, Rhonda K. Yantiss, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-14-0411

Cited articles  This article cites 38 articles, 14 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/8/4/303.full#ref-list-1

Citing articles  This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://cancerpreventionresearch.aacrjournals.org/content/8/4/303.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.