Id2 Drives Differentiation and Suppresses Tumor Formation in the Intestinal Epithelium

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Abstract

Oncogenic signals elevate expression of Id2 in multiple tumor types. When deregulated, Id2 inactivates the tumor suppressor proteins retinoblastoma, p107, and p130. Here, we report a novel and unexpected tumor inhibitory function of Id2 in the intestinal epithelium. First, genetic ablation of Id2 in the mouse prevents differentiation and cell cycle arrest of enterocytes at the time of formation of the crypt-villus unit. Later, these developmental abnormalities evolve toward neoplastic transformation with complete penetrance. Id2-null tumors contain severe dysplastic and metaphasic lesions and express aberrant amounts of β-catenin. Thus, our data are the first to establish a direct requirement of basic helix-loop-helix inhibitors in driving differentiation and define an unexpected role for the retinoblastoma-binding protein Id2 in preventing tumor formation.

Introduction

The basic helix-loop-helix (bHLH) family of transcription factors is essential for development of the secretory lineages of the mouse intestine (goblet, enteroendocrine and Paneth cells; ref. 1). Three cell types are absent in Math1-null mice whereas mice lacking neurogenin3, which acts downstream of Math1, fail to generate enteroendocrine cells (2–4). On the basis of these observations, it was proposed that expression of bHLH transcription factors in intestinal progenitors is required for secretory cell development whereas differentiation toward the alternative enterocytic lineage is inhibited (5).

Members of the Id family (Id1 to Id4) are natural inhibitors of bHLH transcription factors (6). They sequester bHLH proteins and prevent DNA binding of the engaged factor. Numerous data suggested that Id proteins exert negative control on the cell fate decisions and the differentiation pathways initiated by bHLH factors (7, 8). According to this model, it is expected that loss of Id genes result in unrestrained bHLH activity.

Thus far, Id proteins have been viewed as inhibitors of differentiation and positive regulators of cell proliferation and oncogenesis (6, 8). One Id family member, Id2, has additional oncogenic features that distinguish this gene from the other Id family members; it is a direct target of transcription factors of the Myc family and is consequently overexpressed in tumors carrying oncogenic activation of N-myc and c-myc (neuroblastoma and Ewing sarcoma; refs. 9–13). Furthermore, the Id2 protein forms complexes with the retinoblastoma (Rb) protein and, when in stoichiometric excess over Rb, overrides the Rb tumor suppressor function (14, 15).

Recently, several developmental abnormalities have been associated with deletion of the Id2 gene in the mouse (7, 16). In the hematopoietic compartment, Id2-null mice have severe reduction of natural killer cells and lack selected subclasses of dendritic cells (17). They also display defective development of peripheral lymphoid organs. In the mammary gland, Id2-null mice show lactation defects associated with reduced proliferation whereas males have significantly reduced spermatogenesis (7, 16).

Here we show that loss of Id2 in the mouse intestinal epithelium prevents exit from cell cycle and differentiation of enterocyte precursor cells during embryogenesis. The ensuing proliferative activity and loss of differentiation of the intestinal epithelium leads to neoplastic transformation in postnatal life. Thus, our results establish a role for Id proteins in driving lineage-specific differentiation and define a potential tumor suppressor function for inhibitors of bHLH-mediated transcription.

Materials and Methods

Mice and Genotyping. Experiments with animals were done according to Public Health Service guidelines of the Institutional Animal Care and Use Committee at the Albert Einstein College of Medicine, Columbia University and Georgetown University. PCR analysis was conducted to determine the Id2 genotypes as described previously (16).

Histologic Examination and Histochemical Stainings. In all mice, the entire intestinal tract was analyzed as follows. Cuts were made in the intestine every 6 to 8 cm, then the intestine was flushed with formalin to remove the contents. After fixation, the intestine was cut into short segments each approximately 8-cm long. For histologic examination, tumors, areas of intestine with abnormalities of interest, and the coils of intestinal segments covering the entire intestinal tract were processed and paraffin embedded. Sections were cut at 5 μm and stained with H&E. In coiled sections, the location of tumors in the intestine was measured as the distance from the pylorus of the stomach in the coil. Sections of normal intestine and tumors were stained with Alcian Blue (pH 2.5) for mucus-producing goblet cells and with Periodic Acid Schiff, which stains mucus, glycogen, and granules in Paneth cells. In some mice in which tumors were identified by gross examination, part of the tumor and selected regions of the apparently normal intestine were snap frozen in liquid nitrogen for Western blot analysis. Corresponding regions of the intestine from wild-type mice were also frozen. Frozen specimens of adenomas and matched normal intestinal tissue from 4-month-old Min+/− mice were kindly provided by Dr. William Dove, University of Wisconsin, Madison, WI.

Immunohistochemistry. The following antibodies were used: rabbit anti-Id2 (Zymed Laboratories, South San Francisco, CA), mouse anti-β-catenin (BD Biosciences, San Jose, CA), mouse anti-β3-tubulin (Oncogene Research Products, La Jolla, CA), mouse anti-Ki67 (Novocasta, Newcastle, United Kingdom), rabbit anti-chromogranin A (Lab Vision Corporation, Freemont, CA), mouse anti-villin (Chemicon International, Temecula, CA), rabbit anti-cryptdin (kindly provided by Michael Selsted, University of California, Irvine), and rabbit anti-Fabp-1 (kindly provided by Jeffrey Gordon, Washington University, St. Louis). Immunostainings were done according to standard procedures with a biotinylated secondary antibody, followed by streptavidin- HRP, and then developed with 3,3’-diaminobenzidine (brown precipitate). Slides were either lightly counterstained with hematoxylin or no counterstain was applied, depending on the application. For Western blot analysis, lysates were prepared from adenomas and normal intestines using radioimmunoprecipitation assay buffer. After transfer, polyvinylidene difluoride membranes were prepared from adenomas and normal intestines using radioimmunoprecipitation assay buffer. After transfer, polyvinylidene difluoride membranes...
were probed with antibodies against β-catenin (Transduction Laboratories, BD Biosciences) and α-tubulin (Santa Cruz Biotechnology).

**Cell Proliferation.** To measure proliferation of epithelial cells in the intestine, mice received injection with 125 mg/kg BrdUrd, 60 minutes before they were sacrificed. BrdUrd-positive cells were determined by immunohistochemistry in four Ild2−/− mice and four age-matched wild-type mice. The percentage of BrdUrd-positive nuclei in adenomas was calculated by counting the number of nuclei along the entire crypt axis in multiple representative regions. The complete analysis was conducted on four independent adenomas from three Ild2−/− mice. Statistical differences were determined with an unpaired t test (Statview, SAS Institute Inc. Cary, NC).

**Results**

Mutation of Ild2 in the mouse is associated with an intestinal phenotype that may contribute to the retarded growth and the neonatal lethality (16). To address the role of Ild2 during intestine development and function, we first determined expression of Ild2 during development. We examined Ild2 expression by immunohistochemistry before villus development at embryonic day E15.5. Ild2 was expressed in the nuclei of epithelial cells but not in the underlying mesenchyme (Fig. 1A and B). At E18.5, when villus formation is under way, Ild2 was expressed in epithelial cells in the proliferative intervillus region and in the differentiated cells along the villus (Fig. 1C and D). Adult enterocytes exhibited low but detectable expression of Ild2 in the crypt and the villus (data not shown). Next, we followed development and homeostasis of the intestine in Ild2-null mice. No morphological alterations were detected in the Ild2-null primitive intestine of E15.5 embryos, at a developmental age when the gut appears as a tube composed of a pseudostratified epithelium. We identified abnormalities in the small intestine of E18.5 and P0 Ild2-null mice. Nine of nine Ild2 deficient animals at this age contained areas in which villi had been replaced by multilayered, undifferentiated epithelium. These lesions were mostly located in the distal third of the small intestine and were characterized by short, rudimentary villi with broad diameter and blunted tips, lined by cuboidal epithelium in place of the normal columnar enterocytes (Fig. 1E and F). The aberrant villi contained elevated numbers of proliferating Ki67-positive cells in the middle and upper layers of the thickened mucosa (Fig. 1H). This was in contrast with the adjacent normal epithelium and the intestine of wild-type mice that showed Ki67-positive cells only in the crypts and intervillus border (Fig. 1G). The hyperproliferating foci in the intestine of late gestation Ild2−/− embryos and neonates lacked expression of the villus-specific markers villin (ref. 18; Fig. 1I–K) and Fabp-1 (ref. 19; Fig. 1L–N), thus indicating that they retain an undifferentiated phenotype.

Analysis of intestines in adult Ild2−/− mice revealed that the early lesions developed into intestinal tumors with almost complete penetrance. Between the age of 2 and 13 months, 96% of Ild2-null mice (26 of 27) had multiple adenomas (>2.5 tumors per mouse, Table 1). Tumors exhibited a broad base and were classified as tubulovillus (Fig. 2A). The adenomas had elongated, dis-
torted crypts, with hyperplastic mucosa and numerous mitotic figures (Fig. 2B). Some crypts within the adenomas showed dysplastic epithelial changes characterized by closely packed and pseudostratified cells with an increased nuclear-cytoplasmic ratio (Fig. 2C). Localized squamous metaplasia was present occasionally at the base of aberrant crypts (Fig. 2D). Interestingly, regions of the Id2−/− small intestine lacking adenomatous changes exhibited foci of keratinized epithelium that entirely replaced the columnar mucosa whereas retaining a crypt-shaped architecture (Fig. 2E). The neoplastic lesions and mucosal aberrations in the distal ileum herniated in the submucosa and were associated with increased fibrosis that led to macroscopically visible S-shaped distortion of the intestine (Fig. 2F and G; Table 1). These features resulted sometimes in obstructive symptoms and death of the mouse.

The adenomatous areas showed profound differentiation abnormalities of the secretory lineages. Staining for the Paneth cell-specific markers Periodic Acid Schiff (Fig. 3A and B) and cryptidin (Fig. 3C and D; ref. 20) showed that Paneth cells were absent at the base of the adenomas but present in morphologically normal Id2−/− crypts. Compared with normal villi (Fig. 3E), Id2-null adenomas had variable number and distribution of goblet cells. Some tumors had almost no Alcian blue-positive goblet cells (Fig. 3F), but others contained abundant numbers of goblet cells often clustered in distinct groups (Fig.

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**Table 1 Tumor incidence and frequency in the intestine of Id2-null mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Id2−/−</th>
<th>Id2+/-</th>
<th>Id2+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice (no.)</td>
<td>27</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Age (months)</td>
<td>7.6 ± 3.2*</td>
<td>9.3 ± 2.9</td>
<td>5.8 ± 3.1</td>
</tr>
<tr>
<td>Range (months)</td>
<td>2−13</td>
<td>4−13</td>
<td>2−12</td>
</tr>
<tr>
<td>Tumor incidence (%)</td>
<td>96</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tumors/mouse</td>
<td>1−7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mice with distortion in distal ileum (no.)</td>
<td>(2.6 ± 1.9)*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values are mean ± SD.
3G). Staining for the enteroendocrine-specific marker chromogranin showed that enteroneuroendocrine cells were markedly reduced or entirely absent in Id2-null adenomas (Fig. 3H and I).

To ask whether neoplastic changes in this mouse model displayed aberrant proliferation, we injected Id2-null mice with BrdUrd and did quantitative immunostaining analyses. Whereas BrdUrd staining in the normal intestine was limited to the base of the crypt (Fig. 4A), the adenomatous foci had large numbers of proliferating cells along the mid and upper aberrant crypt-villus axis (>4.4-fold increase over the normal crypt-villus unit, P < 0.0001, Fig. 4B and C). The overwhelming majority of intestinal tumors in humans develop genetic alterations leading to stabilization and accumulation of β-catenin (21). Indeed, immunostaining and Western blot analysis showed that all adenomatous lesions of Id2-null mice expressed high levels of cytoplasmic and nuclear β-catenin (Fig. 4D, F, G). Interestingly, β-catenin was moderately elevated in histologically “normal” mucosa from Id2−/− mice (Fig. 4E, G) suggesting that β-catenin levels increased before development of the histologic lesions. The finding of higher expression of β-catenin in normal epithelium from Id2-null intestines contrasted with the undetectable β-catenin signal in the normal mucosa from wild-type and Min+−/− mice (Fig. 4G; ref. 22).

The elevated expression of β-catenin and oncogenic transformation in the Id2-null intestine was unexpected given that Id2 is a direct transcriptional target of β-catenin-TCF and Myc oncoproteins (10, 14, 23, 24). It is intriguing to note that loss of Id2 does not predispose to tumor formation outside the intestine. Conversely, Id2 mediates tumorigenesis in the pituitary gland of Rb+− mice where it is essential for tumor initiation, progression, and angiogenesis.6

Discussion

The results presented here show that Id2 is necessary to secure cell cycle arrest and differentiation of the enterocytic lineage in the developing intestinal epithelium. Through a defined set of events that begin during late mouse embryogenesis, Id2-null intestinal epithelial progenitors fail to undergo terminal differentiation whereas retaining a hyperproliferative state. Later in life, these initial abnormalities evolve toward highly proliferating neoplastic lesions and coexist with an even more complex constellation of differentiation aberrations in the enterocytic and secretory lineages of the intestine.

Earlier studies suggested that bHLH activity is necessary for development and differentiation of the intestinal secretory lineages (5). Our data propose that inhibition of bHLH transcription factors, implemented in a nonredundant fashion by Id2 during embryogenesis, is essential for terminal differentiation of the default enterocytic pathway. Similar models have been proposed to explain the inhibitory function of neurogenic bHLH factors toward the default glial differentiation pathway in the nervous system (25). Thus, the same bHLH proteins might compete in the nervous system and the intestine with glial and enterocyte-specific transcription factors, respectively, for common transcription cofactors (26). However, our results do not exclude that ectopic activation of non-bHLH targets of Id2 contribute to the intestinal phenotype of Id2-null mice (8). The fact that expression of Id2 induces apoptosis might also imply that enterocytes from Id2-null mice are predisposed to tumorigenesis because of their extended survival (27).

Regardless of the molecular mechanism, our study identifies Id2 as another Rb protein target that, like E2F1, can either promote or inhibit cellular transformation (28–31). It is tempting to speculate that certain types of tumor cells may select against Id function to release the activity of specific bHLH or other still unknown Id protein partners whose deregulation in that particular cellular context would carry intrinsic oncogenic activity.

In conclusion, we have assigned for the first time a tumor suppressor function to an inhibitor of transcription factors of the bHLH family. Recent studies showed aberrant expression of the ubiquitous

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6 A. Lasorella, R. Russell, A. Iavarone, personal communication.
bHLH protein ITF2/E2–2 in human cancers with deregulated β-catenin (32) and epigenetic silencing of Id4 through hypermethylation in gastric adenocarcinoma (33). Thus, although the function of bHLH proteins has been mainly linked with positive effects on cell differentiation, we suggest that genetic and/or epigenetic mechanisms leading to ectopic bHLH activity in cells normally requiring their inactivation may represent a powerful initiating event for selected forms of human cancer.

Acknowledgments

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