Suppression of Intestinal Polyposis in \textit{MdrI}-deficient \textit{Apc}\textsuperscript{Min+/−} Mice\textsuperscript{1}

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Abstract

Aberrant transactivation of a certain set of target genes by the \beta-catenin and T-cell factor/lymphoid enhancer factor complex has been considered crucial for the initiation of intestinal tumorigenesis. The human multidrug resistance \textit{Mdr1} (\textit{Abcb1a}) gene contains multiple \beta-catenin–T-cell factor/lymphoid enhancer factor binding elements in its promoter and is one of the immediate targets of the complex. In the current study, we have further substantiated the biological involvement of \textit{Mdr1} in intestinal tumorigenesis based on the following evidence: (a) aberrant induction of the \textit{Mdr1a} (\textit{Abeh1a}) gene product, P-glycoprotein, associated with nuclear accumulation of the \beta-catenin protein, was observed even in nascent microscopic adenomas of Min mice; (b) \textit{Mdr1}-deficient Min (\textit{Apc}\textsuperscript{Min+/−}\textit{Mdr1a/−/−}) mice developed significantly fewer intestinal polyps than did \textit{Apc}\textsuperscript{Min+/−}\textit{Mdr1a+/+} mice; and (c) Inhibitors of P-glycoprotein, verapamil, and cyclosporin A had a suppressive effect on the \textit{in vitro} polyoid growth of IEC6 expressing stabilized (ΔN89) \beta-catenin protein. Inhibitors of P-glycoprotein may be included in a novel class of chemopreventive agents against colorectal carcinogenesis.

Introduction

Mutational inactivation of the tumor suppressor gene \textit{APC}\textsuperscript{3} is the earliest and most frequent genetic event in colorectal carcinogenesis (1). Its inactivation causes IECs to accumulate cytoplasmic \beta-catenin protein and allows the formation of complexes between \beta-catenin and TCF/LEF family transcription factors. Aberrant transactivation of a certain set of target genes by the \beta-catenin and TCF/LEF complexes has been considered crucial for the initiation of intestinal carcinogenesis, and several downstream targets of the complex have already been identified, including c-myc, PPAR-δ, cyclin-D1, TCF-1, c-jun, fra-1, matrylsin (MMP-7), gastrin, ectodermal-neural cortex 1, laminin \gamma2, ITF-2, and axin2 (Axil) (2–7). We demonstrated previously that the human \textit{Mdr1} \textit{(Abcb1)} gene contains multiple \beta-catenin–TCF4-binding elements in its promoter and is an immediate target of the complex (8). These target genes are likely to be important mediators of intestinal carcinogenesis and good candidates for chemoprevention of colorectal cancer by molecular targeting. However, because with a few exceptions (3, 9–11) the functional connection of these downstream target genes with intestinal carcinogenesis has remained unexplored, in this study, we adopted a genetic approach to clarify the functional involvement of the \textit{Mdr1} gene in intestinal tumorigenesis. We report suppression of intestinal tumorigenesis in \textit{Apc}\textsuperscript{Min+/−} mice lacking functional \textit{Mdr1} genes.

Materials and Methods

Antibodies, Immunoblot Analyses, Immunohistochemistry, and Immunofluorescence Microscopy. Anti-\beta-catenin monoclonal antibody (clone 14) was purchased from BD Transduction Laboratories (San Diego, CA). Anti-\beta-catenin (C-18), anti-MDR (C-19 and H-241), and anti-actin (C-11) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Immunoblot analyses and immunohistochemistry were performed essentially as described previously (8). For immunofluorescence microscopy, cells were grown on type-I-collagen-coated coverslips (Asahi Technoglass, Tokyo, Japan), fixed with 10% buffered formalin, and incubated with anti-\beta-catenin monoclonal antibody overnight. After incubation with Alexa Fluor 488-labeled goat antimouse IgG (Molecular Probes, Eugene, OR), the coverslips were inspected with a laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Reverse Transcription-PCR. Total RNA was prepared from adrenal gland, normal small intestine, and polyp tissues of Min mice (C57BL/6J-\textit{Apc}\textsuperscript{Min+/-}) with TRIzol (Invitrogen, Carlsbad, CA). A 1-μg sample of DNase-I-treated total RNA was reverse transcribed and amplified by PCR. The following PCR primers were used: for \textit{Mdr1a}, 5′-TGAAGAGTGTGAATCTAAGGAT-3′ and 5′-TGAAGAATAATGATCCCAAGGAT-3′; for \textit{Mdr1b}, 5′-TAAATGTTATGATCCCAAGT-3′ and 5′-TTTATGTCATCCTCTTGTA-3′; for \textit{Mdr3}, 5′-ATTTGGAGTTGAGCTAAGACGGAGC-3′ and 5′-ATAGCTATCTCAGACGAGGA-3′; for cytokeratin 19, 5′-TTGAGATTGAGTCGTCCACACTG-3′ and 5′-TTCCAGGGGAGCTCCTGTC-3′; for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), rodent GAPDH forward and reverse primers (Applied Biosystems (Foster City, CA). PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

\textit{Mdr1} and \textit{Apc} Compound Mutant Mice. Animal experiments were performed in accordance with the guidelines of the National Cancer Center Research Institute (Tokyo, Japan). C57BL/6J male Min (\textit{Apc}\textsuperscript{Min+/−}) mice (12) were obtained from The Jackson Laboratory (Bar Harbor, ME), and FVB female \textit{Mdr1a/b} double knockout mice (\textit{Mdr1a+/−/Mdr1b+/−}; Ref. 13) were obtained from Taconic Farms (Germantown, NY). The genotyping of the animals was described in Fig. 2 and its legends. N0F1 pups were housed under identical conditions and sacrificed at 15 weeks of age. The gut was filled with 10% buffered formalin via the anus immediately after sacrifice and then opened longitudinally. After overnight fixation, specimens were stained briefly with 0.5% methylene blue. The numbers and major diameters of polyps in the small and large intestine were measured in the ×20 power field of a dissecting microscope (Nikon, Tokyo, Japan). Formalin-fixed, paraffin-embedded sections were stained by standard H&E techniques.

Cell Lines and Tetracycline-inducible Retroviral Expression. An immortalized rat small cell line, IEC6 (14), was obtained from the Riken Cell Bank (Tsukuba, Japan) and cultivated in DMEM containing 5% Tet-system-mortalized rat small cell line, IEC6 (14), was obtained from the Riken Cell Bank (Tsukuba, Japan) and cultivated in DMEM containing 5% Tet-system.
Louis, MO) with conditioned medium containing ecotropic retroviral particles, as described previously (16) and cloned by limiting dilution with uninfected cells as feeder layers.

To visualize cross-sections of polypoid foci, cells were cultured on type-I-collagen (Koken, Tokyo, Japan)-coated PeriPERM dishes (Vivascience, Hanover, Germany) for 4 weeks. Glutaraldehyde and osmic acid-fixed cells were dehydrated in a graded ethanol series and then embedded in LR-white resin. Thin sections (4–6 μm) were cut and stained by standard toluidine blue techniques.

**Chemicals.** Dox (Sigma) was dissolved in deionized water to a stock concentration of 1 mg/ml. Dox was added to the culture medium to a final concentration of 0.01 μg/ml for maintenance and to 0.1 μg/ml for suppression of induction. R(+)-verapamil and cyclosporin A (Sigma) were dissolved in ethanol to obtain a stock concentration of 20 and 5 mM, respectively, and added to the culture medium in the range of 5–100 μM and 0.5–10 μM, respectively. The culture media containing these drugs were replaced every 2 or 3 days.

**Dual Luciferase Reporter Assay.** We used a pair of luciferase reporter constructs, TOP-FLASH and FOP-FLASH (Upstate, Placid, NY), to evaluate TCF/LEF transcriptional activity (16). Cells were transiently transfected in triplicate with one of these luciferase reporters and phRL-TK (Promega, Madison, WI) by using FuGENE 6 transfection reagent (Roche, Mannheim, Germany). Luciferase activity was measured with the Dual-luciferase reporter assay system (Promega), 72 h after transfection, with Renilla luciferase activity as an internal control.

**Results and Discussion**

**Increased Expression of P-Glycoprotein in Intestinal Adenoma of Min Mice.** To substantiate the involvement of the MDR1 gene in intestinal tumorigenesis, we first examined the expression of Mdr1 in intestinal polyps of Min (C57BL/6J-Apc<sup>Min<sup>−/−</sub></sup>) mice. Min mice have a
germ-line mutation at codon 850 of the Apc gene and spontaneously develop numerous adenomatous polyps in the small and large intestine (12), and for that reason, they are recognized as an appropriate animal model of human FAP syndrome. Immunohistochemical analysis of intestinal adenomas of Min mice revealed that all of the adenoma cells examined contained higher amounts of P-glycoprotein, the Mdr gene product, than neighboring normal IECs (Fig. 1, C and D). The increased expression of P-glycoprotein was always associated with nuclear accumulation of β-catenin protein as determined by staining serial sections with anti-β-catenin antibody (Fig. 1, A and B) and anti-P-glycoprotein antibody (Fig. 1, C and D). Aberrant expression of P-glycoprotein was evident even in a nascent microadenoma formed within a single villus (Fig. 1F), supporting the hypothesis that induction of the Mdr gene is an early event in adenoma development. These results are consistent with our previous observations in human specimens (8).

The anti-P-glycoprotein polyclonal antibody used for immunohistochemistry is known to react with the products of the Mdra1 (Abcb1a, P-glycoprotein 1), Mdra2 (Abcb1b, P-glycoprotein 2), and Mdra3 (Abcb4, P-glycoprotein 3) genes. Reverse transcription-PCR using primers specific for Mdra1, Mdra2, and Mdra3, respectively (Fig. 1G), revealed exclusive expression of Mdra1 in the intestine of Min mice. Consistent with immunohistochemical analyses, Mdra1 mRNA was found to be up-regulated in tumor tissue when compared with normal intestine.

**Suppression of Intestinal Tumorigenesis in Mdra1b-deficient ApcMin/+ Mice.** Next, we generated compound mutant mice by introducing a homozygous deletion mutation of the Mdra1a and Mdra1b genes into the APCMin/+ heterozygotes to validate the functional involvement of Mdra1 in β-catenin-mediated colorectal carcinogenesis. Although Mdra1b, a sister gene of Mdra1 in rodents, is not expressed in intestinal adenoma of Min mice (Fig. 1G), both the murine Mdra1a and Mdra1b genes contain two consensus sequences of TF/C/LEF binding, CTTTGA/FAT, in their promoters (17, 18). To avoid compensation by unexpected transactivation of the Mdra1b gene, we used Mdra1a and Mdra1b double-knockout mice, instead of Mdra1a single-knockout mice. No clear phenotypical abnormalities have been reported in mutant mice lacking the Mdra1a and Mdra1b genes (13).
Mdr1a/b-knockout mice were generated in the FVB strain, as described previously (13). Different genetic backgrounds are thought to be capable of altering the severity of the Min phenotype, and one known modifier of the Min phenotype is the Mom1 locus, which contains the secretory phospholipase A2 (Pla2g2a) gene (19). The FVB strain carried only dominant wild-type alleles for the Pla2g2a gene (+/+/+/), considered to be resistant to the Min phenotype; Fig. 2, E and F), whereas Min (C57BL/6J-ApcMin+) mice carry only recessive mutated alleles (−/−, sensitive to the Min phenotype). To replace Mom1 and the other possible unknown modifiers in the FVB strain and unify the genetic background, female FVB-Mdr1a/b−/− mice were backcrossed repeatedly to male C57BL/6J-ApcMin+ or C57BL/6J mice to the N6 generation. The resulting genetically coordinated N6 male ApcMin+/+/+/−Mdr1a/b+/− mice and N6 female Apc−+/+/+/−Mdr1a/b−/− mice were mated to produce N6F1 pups. ApcMin+/+ N6F1 mice were assessed for intestinal polyposis according to their Mdr1a/b genotypes. Because of their close physical distance, we never observed dissociation between the Mdr1a and Mdr1b loci in any of the experiments. 4 The number of polyps in the small intestine of ApcMin+/+/+/−Mdr1a/b+/− mice was reduced to almost half the number in the control ApcMin+/+ Mdr1a/b−/− mice (Fig. 3, A and B; average 102.1 ± 55.7/male mouse, P = 0.0021; 147.1 ± 74.4/female mouse, P = 0.0041). The ApcMin+/+ female N6F1 mice tended to have more polyps than the males.

4 A. H. Schinkel, personal communication.
implying the presence of a gender-related weak modifier(s), probably derived from the FVB strain. There were no significant differences between the Mdr1a/b−/− and Mdr1a/b+/− mice in the major diameters or locations of the polyps, a finding consistent with the recently proposed “hit-and-run” theory of the role of mutated β-catenin, in which the β-catenin–TCF4 complex plays a major role only in the initiation of tumorigenesis (20). A similar reduction in polypl number was observed in the colon of male Nf1 ApcMin/+ Mdr1a/b−/− mice (Mdr1a/b+/−, 4.3 and Mdr1a/b−/−, 2.3 mm on average) but not in female mice (Mdr1a/b+/−, 3.6 and Mdr1a/b−/−, 3.2). However, because of the general paucity of polyps in the colon, it would be premature to draw conclusions.

P-glycoprotein, encoded by the MDRI gene, is a member of the superfamily of ABC transporters that transfer various molecules across membranes. Human ABC genes are divided into eight distinct subfamilies: (a) ABC1; (b) MDR/TAP; (c) MRP; (d) ALD; (e) OABP; (f) GCN20; (g) White; and (h) ANSA, and the MDRI gene belongs to the MDR/TAP subfamily (21). The incomplete inhibition of intestinal polyposis in the absence of the Mdr1a/b genes may be attributable to compensation by other member(s) of the ABC transporter superfamily.

In addition to the reduction of polypl numbers, we noted morphological alterations in the polyps of Mdr1a/b-deficient mice (Fig. 3, C and D). The intestinal polypls of Mdr1a/b−/− mice tended to be flat, and their top surface was centrally depressed and often covered with necrotic material (Fig. 3C). Histological examination of the polypls (Fig. 3D) revealed detachment of the covering non-neoplastic epithelium. Similar morphology has been reported in the polypls of ApcMin-Alk mice deficient in prostaglandin receptor EP2 or cyclooxygenase-2 (22, 23). An association between MDRI and expression of cyclooxygenase-2 has been documented recently (24, 25). Cytosolic phospholipase A2 releases lyso-PAF, a precursor of PAF, from plasma membrane lipid layers as a coproduct of arachidonic acid. PAF is one of the substrates of P-glycoprotein.
Overexpression of P-glycoprotein may directly or indirectly influence the release of arachidonic acid and subsequent production of prostanooids. The precise mechanism of the suppression of intestinal polyposis in Mdr1-deficient mice, however, remains to be elucidated.

Establishment of a Rat IEC Clone Capable of Inducing Stabilized β-Catenin. Lastly, to clarify the role of Mdr1 in early stage intestinal tumorigenesis, we used a tetracycline-regulatory system to engineer an immortalized rat IEC line, IEC6. Native IEC6 cells maintain contact inhibition and anchorage dependency and do not display tumorigenicity in nude mice (27). IEC6 cells are phenotypically similar to undifferentiated normal intestinal cryptic cells and retain the potential to differentiate into various cell lineages, including absorptive enterocytes, goblet cells, Paneth cells, and endocrine cells (28). We used retroviral gene transfer and limiting dilution to establish a stable clone, IEC6-TetOFF β-catenin DN89, that is capable of inducing stabilized β-catenin protein (15) on removal of Dox from the culture medium (Fig. 4A, left) and a mock clone, IEC6-TetOFF control (Fig. 4A, right). Induction of β-catenin DN89 caused TCF/LEF-specific gene transactivation, as revealed by luciferase reporter assays (Fig. 4B), and nuclear and cytoplasmic accumulation of the β-catenin protein, as revealed by immunofluorescence microscopy (Fig. 4C).

IEC6-TetOFF β-catenin DN89 cells developed numerous polypoid foci when maintained confluent for 2–4 weeks in the absence of Dox (with induction of the β-catenin DN89 protein; Fig. 4D, right). IEC6-TetOFF β-catenin DN89 cells maintained a flat monolayer in the presence of Dox (without induction of the β-catenin DN89 protein; Fig. 4D, left), similar to native IEC6 cells. We reported previously that induction of a dominant-negative form of TCF4 or sulindac, a clinically proven chemopreventive agent against polyposis in FAP patients, suppressed the similar polypoid growth of a colorectal cancer cell line, DLD1 (16). The formation of polypoid foci in vitro is thus confirmed to reflect the adenomatos proliferator of IECs.

Vertical cross-sections revealed that the polypoid foci were shaped by cells migrating underneath the flat cell monolayer (Fig. 4E, bottom; Dox (−)), and those cells exhibited enlarged nuclei and nucleoli. Abnormal migration of IEC6 cells expressing the stabilized β-catenin protein may imitate the inward migration of IECs, which is believed to give rise to nascent microadenoma (29).

Suppressive Effects of P-Glycoprotein Inhibitors on in Vitro Polypoid Growth. On induction of β-catenin DN89 protein, IEC6 cells increased their expression of P-glycoprotein (Fig. 4F), and we examined the effect of two well-characterized inhibitors of P-glycoprotein, verapamil and cyclosporin A (30), on the polypoid growth of IEC6 cells expressing β-catenin DN89 protein. The addition of a minimum of 40 μM verapamil or 1.5 μM cyclosporin A to the culture medium almost completely suppressed polypoid growth (Fig. 4G). Cyclosporin A (data not shown) and verapamil exerted similar suppressive effects on the polypoid growth of DLD1 cells (Fig. 4H).

Current chemoprevention by NSAIDs alone does not seem to be a satisfactory means of suppressing tumorigenesis in FAP patients and groups at high risk of colorectal cancer (31). Several newer generation P-glycoprotein antagonists, such as PSC-833 (Valspodar) and MS-209, have been developed with the intention of reversing MDR in cancer cells (32), and these drugs may be diverted to chemopreventive uses against colorectal cancer. The addition of these P-glycoprotein inhibitors may improve the chemopreventive efficacy of the current protocol using NSAIDs alone by mechanisms different from those of NSAIDs.


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