Genetic Ablation of M₃ Muscarinic Receptors Attenuates Murine Colon Epithelial Cell Proliferation and Neoplasia

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Abstract

Colon epithelial cells express and most colon cancers overexpress M₃ muscarinic receptors (M₃R). In human colon cancer cells, post-M₃R signaling stimulates proliferation. To explore the importance of M₃R expression in vivo, we used the azoxymethane-induced colon neoplasia model. Mice treated with weekly i.p. injection of saline [10 wild-type (WT) mice] or azoxymethane (22 WT and 16 M₃R⁻/⁻ mice) for 6 weeks were euthanized at 20 weeks. At week 20, azoxymethane-treated WT mice weighed ∼ 16% more than WT M₃R⁻/⁻ mice (33.4 g ± 1.0 g versus 27.9 g ± 0.5 g; mean ± SE, P < 0.001). In azoxymethane-treated M₃R⁻/⁻ mice, cell proliferation (BrdUrd staining) was reduced 43% compared with azoxymethane-treated WT mice (P < 0.05). Whereas control mice (both WT and M₃R⁻/⁻) had no colon tumors, azoxymethane-treated WT mice had 5.3 ± 0.5 tumors per animal. Strikingly, azoxymethane-treated M₃R⁻/⁻ mice had only 3.2 ± 0.3 tumors per mouse (P < 0.05), a 40% reduction. Tumor volume in azoxymethane-treated M₃R⁻/⁻ mice was reduced 60% compared with azoxymethane-treated WT mice (8.1 mm³ ± 1.5 mm³ versus 20.3 mm³ ± 4.1 mm³; P < 0.05). Compared with WT, fewer M₃R⁻/⁻ mice had adenomas (6% versus 36%; P = 0.05), and M₃R⁻/⁻ mice had fewer adenocarcinomas per mouse (0.6 ± 0.1 versus 1.7 ± 0.4; P < 0.05). Eleven of 22 WT but no M₃R⁻/⁻ mice had multiple adenocarcinomas (P < 0.001). Compared with WT, azoxymethane-treated M₃R-deficient mice have attenuated epithelial cell proliferation, tumor number, and size. M₃R and post-M₃R signaling are novel therapeutic targets for colon cancer.

Introduction

The muscarinic cholinergic family of G protein–coupled receptors includes five muscarinic receptor subtypes designated M₁ to M₅ (1–3). M₃ muscarinic receptors (M₃R) are expressed widely in the gastrointestinal tract, couple to Gi/11, activate phospholipase C signaling and inositol phosphate formation, and increase cell calcium, thereby altering cell function, including proliferation (4). In 1991, Gutkind et al. (5) reported that muscarinic receptors linked to phosphatidylinositol hydrolysis (i.e., M₁R, M₂R, and M₃R) act as conditional oncogenes when expressed in cells capable of proliferation. Subsequently, Frucht and colleagues (6, 7) reported that human colon cancer cell lines and colon cancer tissue express M₃R, and that M₃R expression was increased up to 8-fold in cancer compared with normal tissue. Collectively, these findings suggest that the M₃R is an important player in colon neoplasia.

Work from our laboratory elucidated cellular mechanisms underlying acetylcholine-induced colon cancer cell proliferation. In H508 human colon cancer cells that express high levels of M₃R (8), acetylcholine activates postreceptor signaling, including robust phosphorylation of extracellular signal–regulated kinase (ERK)1/2 and p90RSK, a nuclear response protein that regulates gene expression and the cell cycle (9). These actions of acetylcholine are blocked by muscarinic receptor inverse agonists, calcium chelators, and inhibitors of mitogen-activated protein/ERK kinase (the regulatory protein just upstream of ERK), and they do not occur in SNU-C4 human colon cancer cells that do not express M₃R (9). Moreover, recent studies support the hypothesis that luminal bile acids, long associated with the development of colon cancer (10), also stimulate colon cancer cell proliferation by M₃R- and epidermal growth factor receptor–dependent mechanisms (11–13). In these in vitro cell systems, proliferative actions of muscarinic agonists require both M₃R expression and activation of post-M₃R signaling.

Given the key role of M₃R expression in proliferation of human colon cancer cells, it is important to determine whether genetic ablation of M₃R reduces colon tumor formation in vivo. In rodents, azoxymethane (10 mg/kg body weight), an intermediate in the metabolism of 1,2-dimethylhydrazine to the alkylating ion methylazldionium, is a colorectal-specific procarcinogen (14). Azoxymethane-induced rodent tumors mimic human colon cancer; most lesions arise in the colon, form grossly visible exophytic polyoid or plaque-like tumors, and have a microscopic appearance similar to that of human adenomas and adenocarcinomas. Molecular changes in azoxymethane-induced tumors, including β-catenin and p53 mutations, also mimic those in human colon cancer (15, 16). We used the azoxymethane colon cancer model in M₃R-deficient mice to determine whether M₃R expression is required for azoxymethane-induced cell proliferation and neoplasia (14). As reported herein, our findings strongly support the hypothesis that M₃R plays a key role in colon epithelial cell proliferation and neoplasia.

Materials and Methods

Animals. The generation of M₃R⁻/⁻ and wild type (WT) mice of the same mixed genetic background (C57BL/6J × 129S6/SvEvTac CF1; 50%/50%) has been described previously (17). For all experiments, male mice were used. Mice were housed under identical conditions in a pathogen-free room, had free

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access to commercial rodent chow and water, and were allowed to acclimatize in the vivarium for 2 wk before initiating treatments. Mice were weighed weekly. These studies were approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee.

In situ hybridization. As described previously (18), digoxigenin-labeled antisense RNA probes were prepared from riboprobe plasmids containing M1R and M3R inserts. M1R riboprobe, synthesized from a 0.28-kb KpnI-Sacl genomic fragment cloned into a pBluescript vector, corresponds to the M1R sequence lacking in the genome of M1R/C0/C0 mice. M3R riboprobe, synthesized from a 1.6-kb XbaI-Sse8337I genomic fragment, corresponds to M3R sequence absent in the genome of M3R/C0/C0 mice. M1R and M3R riboprobes were digested with KpnI and NotI, respectively. After purification of linearized plasmids, in vitro transcriptions for M1R and M3R RNA probes, 280 bp and 1.6 kb in length, respectively, were performed using the Digoxigenin RNA labeling kit (Roche Applied Sciences) with T7 and T3 RNA polymerases, respectively. The length of M3R digoxigenin-labeled RNA was shortened by alkaline hydrolysis to ~300 bp. The yield of transcripts was estimated using dot blots with control digoxigenin-labeled RNA (Roche Applied Science).

Azoxymethane treatment. For the initial 6 wk of treatment, 48 animals received weekly i.p. azoxymethane (Midwest Research Institute; 10 mg/kg body weight; 22 WT and M3R/C0/C0 mice) or PBS (10 WT mice). To determine tumor incidence, mice were observed for evidence of tumor formation (e.g., anal bleeding) and euthanized at 20 wk. Colon length was measured, segments opened longitudinally, and placed flat on microscope slides. Tumors were identified by visual inspection and photographed using a dissecting microscope (Nikon SMZ1500). Tumor size was measured using calipers and confirmed using Nikon Image-Pro software. Tumor volume was calculated using the equation: volume = 1/2 (length \times width)2 (ref. 19).

Histologic analysis. Adenomas and adenocarcinomas were defined according to consensus recommendations (Mouse Models of Human Cancers Consortium; ref. 14). Tumor number was counted in the colon from each mouse. Tumors were photographed, resected, and bisected. Tissues were fixed in 4% paraformaldehyde and paraffin embedded. Five-micrometer sections were stained with H&E and examined using a Nikon Eclipse 80i microscope. Investigators, who were masked to mouse genotype and treatment, performed gross and microscopic tumor counts, and determined tumor size.

Immunohistologic analysis. Two hours before euthanasia, mice received i.p. injection of BrdUrd (Sigma-Aldrich; 50 mg/kg) to label S-phase cells, a marker of proliferation. BrdUrd labeling was determined after immunostaining with anti-BrdUrd antibody (BD Bioscience) by counting BrdUrd-positive nuclei in 1,000 cells (data expressed as percentage of total cells that were BrdUrd positive). As a marker of apoptosis, we used immunostaining with anti–activated caspase-3 antibody (Cell Signaling Technology; ref. 20). Only complete crypts were evaluated, and investigators were masked to treatment group. For analysis of both BrdUrd and activated caspase-3 staining, only tissue from the distal half of the colon was examined.

Statistical analysis. Based on distribution of the data, Student’s unpaired t test (normally distributed data) or the Mann-Whitney U test (nonparametric data) were used to determine significance. A one-way ANOVA with Tukey HSD test was performed to compare three or more groups. Nominal data were analyzed using \( \chi^2 \) with Fisher’s exact test. \( P \) values of <0.05 were considered significant. Statistical analysis was performed using StatView (SAS, version 5.0.1).

Figure 1. WT and M3R/C0/C0 mouse colon mucosal histology and muscarinic receptor expression, study protocol, and animal weights. A, representative H&E-stained colon sections from WT and M3R/C0/C0 mice. B, in situ hybridization using specific M1R and M3R riboprobes in colon epithelium from WT and M3R/C0/C0 mice. C, schematic of study design; WT and M3R/C0/C0 mice were treated with i.p. injection of PBS (10 WT mice) or azoxymethane (22 WT and 16 M3R/C0/C0 mice) weekly for 6 wk and followed for a total of 20 wk. At 20 wk, animals were euthanized and colon tumor number and size, and mucosal markers of proliferation and apoptosis were measured. D, weights of PBS-treated WT, and azoxymethane-treated WT and M3R/C0/C0 mice during the 20-wk study (mean ± SE).
Results

Deletion of M3R does not perturb normal gastrointestinal development (17). As shown in Fig. 1A, there was no difference in microscopic anatomy in H&E-stained colon sections from WT compared with those from M3R−/− mice. To confirm expression of M3R and identify possible coexpression of M3R in colon epithelial cells, we used in situ hybridization with muscarinic receptor-specific riboprobes. Coexpression of M3R and M3R was previously detected in WT murine gastric mucosa (18). As expected, whereas signals for both M3R and M3R were evident in colon epithelial cells from WT mice, only M3R signal was detected in M3R−/− animals (Fig. 1B). To determine the effect of M3R deficiency on colon tumor formation, 48 animals were allocated to weekly i.p. injection of azoxymethane (22 WT and 16 M3R−/− mice) or PBS (10 WT mice) for a total of 6 doses each (Fig. 1C). Animals were euthanized at 20 weeks to measure tumor number and size, and markers of proliferation and apoptosis (Fig. 1C). As reported previously (17), at baseline, M3R−/− weighed less than WT mice (20.7 grams F 0.5 grams versus 28.3 grams F 0.5 grams; mean F SE; P < 0.001).

Over the course of the 20-week study, WT mice treated with azoxymethane gained less weight than WT mice treated with PBS (33.4 grams F 1.0 grams versus 38.7 grams F 0.6 grams at week 20; P < 0.01; Fig. 1D). Whereas animals treated with PBS gained weight progressively over the 20-week study, animals treated with azoxymethane did not gain weight during the initial 6 weeks but gained weight once the series of azoxymethane injections was completed (Fig. 1D). Moreover, although both groups gained weight, azoxymethane-treated WT mice persistently weighed ~16% more than M3R−/− mice (33.4 grams ± 1.0 grams versus 27.9 ± 0.5 grams at week 20; P < 0.001; Fig. 1D). Colons were modestly, but significantly, shorter in M3R−/− compared with WT mice (10.7 cm ± 0.2 cm versus 11.6 cm ± 0.2 cm for M3R−/− compared with WT mice treated with azoxymethane; P = 0.002). Before euthanasia, no mice developed rectal bleeding or other clinical evidence of colon tumor formation.

As a marker of cell proliferation, BrdUrd (50 mg/kg) was administered by i.p. injection 2 hours before euthanasia. Figure 2A shows representative micrographs of BrdUrd incorporation in colon mucosa from PBS-treated WT, and azoxymethane-treated WT and M3R−/− mice. Azoxymethane treatment induced BrdUrd incorporation in normal colon crypts (Fig. 2A). A 5-fold increase in BrdUrd-positive epithelial cells was detected in tissue from azoxymethane-treated WT mice and this was reduced in M3R−/− animals (Fig. 2A and B). Figure 2B shows an ~70% reduction in BrdUrd-positive cells in tissue from M3R−/− compared with WT mice (7.2% ± 0.9% versus 16.6% ± 4.0%; P < 0.05). Clearly,

Figure 2. Markers of cell proliferation and apoptosis in colon epithelial cells from PBS- and azoxymethane-treated WT and M3R−/− mice. A, representative BrdUrd-stained sections from PBS-treated WT, azoxymethane (AOM)-treated WT, and azoxymethane-treated M3R−/− mice. Arrows, representative BrdUrd-stained nuclei. B, percentage of BrdUrd-positive cells in colon epithelial cells in the three treatment groups. Columns, mean percentage BrdUrd-stained cells; bars, SE. n = 5 animals per group. Unpaired Student’s t test. C, representative activated caspase-3-stained sections from PBS-treated WT, azoxymethane-treated WT, and azoxymethane-treated M3R−/− mice. Arrows, activated caspase-3–stained cells. D, percentage of activated caspase-3–positive cells in colon epithelial cells in the three treatment groups. Columns, mean percentage activated caspase-3–stained cells; bars, SE. n = 5 animals per group. Unpaired Student’s t test.
Azoxymethane treatment stimulates proliferation of normal colon epithelial cells and this effect is attenuated in M3R-deficient mice (Fig. 2B).

As a marker of apoptosis, sections of colon mucosa from PBS-treated WT, and azoxymethane-treated WT and M3R−/− mice were examined for activated caspase-3 immunostaining (representative micrographs shown in Fig. 2C). As depicted in Fig. 2D, the number of apoptotic cells in the three treatment groups was an order of magnitude lower than observed with BrdUrd staining. There was no significant difference in the number of apoptotic cells between groups (P > 0.05). These findings indicate that azoxymethane primarily stimulates epithelial cell proliferation and that genetic ablation of M3R reduces cell proliferation with no appreciable effect on apoptosis.

Representative colon sections from azoxymethane-treated animals shown in Fig. 3A indicate reduced tumor number in azoxymethane-treated M3R−/− compared with WT mice. At 20 weeks, no colon tumors were observed in WT mice that had not been treated with azoxymethane. Likewise, colon tumors were not observed in M3R−/− mice that were not treated with azoxymethane (data not shown). As illustrated in Fig. 3B, WT mice treated with azoxymethane had 5.3 ± 0.5 tumors per colon, whereas M3R−/− mice had 3.2 ± 0.3 tumors per colon; a 40% reduction (P < 0.05). In both WT and M3R-deficient mice, all tumors were in the distal half of the colon. Tumor volume was reduced by 60% in M3R−/− compared with WT mice (8.1 mm³ ± 1.5 mm³ versus 20.3 mm³ ± 4.1 mm³; P < 0.05; Fig. 3C). These findings provide strong evidence that M3R gene ablation decreases both colon tumor number and size.

Azoxymethane-induced colon tumors mimicked the gross (Fig. 4A) and microscopic (Fig. 4B) appearance of human adenomas and adenocarcinomas. Tumors observed in azoxymethane-treated mice included adenomas (Fig. 4B, left), well-differentiated adenocarcinomas (data not shown), and poorly differentiated adenocarcinomas (Fig. 4B, right). Histologically, these tumors revealed the characteristic features of early adenomas, including neoplastic mucin production and condensation of colonic crypts. In addition, there was a marked increase in the number of azoxymethane-induced tumors in M3R−/− mice as compared with WT mice (Fig. 4B). These findings strongly suggest that M3R plays a critical role in the development of azoxymethane-induced colon tumors.
adenocarcinomas (Fig. 4B, middle and right). To determine the relative number of adenomas and adenocarcinomas, H&E-stained colon sections were reviewed by a pathologist masked to treatment group (22 WT and 16 M3R−/−/C0 mice treated with azoxymethane). This analysis revealed that, compared with WT, fewer M3R−/− mice had adenomas (6% versus 36%; P = 0.05) and M3R−/− mice had fewer adenocarcinomas per mouse (0.6 ± 0.1 versus 1.7 ± 0.4; P < 0.05; Fig. 4C). Eleven of 22 WT but no M3R-deficient mice had multiple adenocarcinomas (P < 0.001). Collectively, these findings indicate that in this murine model of colon cancer, M3R-deficiency attenuates the overall number of colon tumors and, more specifically, the number of adenomas and adenocarcinomas.

Discussion

In the United States, colon cancer is a common disease that kills ~50,000 people each year.4 Prevention and early detection by screening, with endoscopic removal of colon adenomas, is effective. However, advanced, nonresectable lesions do not respond well to chemotherapy or radiation. Hence, targeting growth factors and growth factor receptors (e.g., M3R) remains an important therapeutic strategy. The present study was designed to evaluate the role of M3R in colon neoplasia and to determine whether M3R is a potential target for colon cancer treatment.

Using a well-established in vivo model of colon neoplasia and M3R-null mice, we provide strong evidence that M3R, which are commonly expressed in human colon cancer (7), play a key role in colon neoplasia. In both gross and microscopic appearance, colon tumors in azoxymethane-treated animals mimicked human disease (Fig. 4). Our findings reveal that in azoxymethane-treated mice, genetic ablation of M3R attenuates epithelial cell proliferation, and the number of adenomas and adenocarcinomas per mouse colon (65% reduction in the number of adenocarcinomas per colon). Whereas 50% of azoxymethane-treated WT animals had multiple adenocarcinomas per colon, this was not the case with any M3R-deficient animal. Moreover, in M3R-deficient mice, the overall colon tumor volume was reduced by 60% compared with that in WT animals. In the colon, potential muscarinic receptor agonists include acetylcholine that is released by enteric neurons or produced by colon cancer cells as autocrine growth factors.5 Bile acids that are reported to increase colon cancer risk also interact functionally with M3R (11–13). Collectively, these observations suggest that M3R may play a role in both tumor initiation and promotion; that is, both the number and size of tumors was reduced in M3R-deficient animals. Hence, in addition to being a growth factor receptor, M3R may play an important role in initiation of colon neoplasia.

In general, treatment with azoxymethane stimulated a robust increase in epithelial cell proliferation (Fig. 2A and B). In contrast, there was little apoptosis in colon sections obtained from animals in any treatment group (Fig. 2C and D). Neither treatment with azoxymethane nor ablation of M3R affected epithelial cell apoptosis as measured by the appearance of activated caspase-3. Therefore,

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the role of M3R in promoting colon neoplasia seems to depend primarily, if not solely, on proliferative signaling. This observation is consistent with our in vitro observations in human colon cancer cells that post-M3R/EGFR signaling is mediated primarily by the proliferative ERK pathway and that blocking ERK signaling abolishes M3R-agonist–induced cell proliferation (8, 11–13).

From the present data, we cannot comment on the role that coexpression of M1R and M3R in colon epithelial cells plays in colon neoplasia. Previous work indicates that expression of M1R does not increase in M3R-deficient mice, at least not in those tissues studied (17). Because M1R and M3R have a similar G protein coupling profile (2, 3), it is possible that M1R have a similar functional role as M3R in colon epithelial cells (18). This issue can be addressed in future studies by using mice that are deficient in both muscarinic receptor subtypes (18).

Finally, our findings have potentially important implications regarding reducing expression or activation of M3R to prevent or treat colon cancer in humans. Nonetheless, practical translation to clinical trials of the observations presented herein requires demonstration that targeting M3R and/or downstream signaling with chemical inhibitors mimics genetic ablation of M3R, thereby attenuating colon neoplasia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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