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Muscarinic acetylcholine receptors participate in small intestinal mucosal homeostasis

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ABSTRACT

Background: Intestinal mucosal homeostasis is controlled by multiple factors and an intact, functional mucosa is essential for survival. Maintenance of the epithelium begins with crypt base stem cells which eventually give rise to all epithelial cell types. Evidence suggests an important role of the enteric cholinergic nervous system in these processes. We hypothesized that mice with altered muscarinic signaling would exhibit differences in mucosal morphometric and proliferative parameters compared to wild-type mice.

Methods: Mouse lines specifically deficient in one of the five muscarinic acetylcholine receptors (M1KO-M5KO) were used for experiments. Distal ileal segments were obtained and histologic sections created. Villus height and crypt depth were measured using H&E-stained sections, while crypt proliferation index (CPI) was calculated using Ki67-stained sections.

Results: The ileal mucosa from mice deficient in mAChRs exhibited differences from wild-type ileal mucosa in nearly all measured parameters. Knockout of mAChR2, mAChR3 and mAChR5 resulted in changes in all measured parameters. Ileal mucosa from M2KO mice showed an unexpected combination decreased VH but paradoxically increased CD and CPI.

Conclusions: Alterations in mAChR signaling causes change in ileal mucosal morphometry and crypt cell proliferation. While all mAChR subtypes may be involved, mAChR2, mAChR3, and mAChR5 appear to be critical for mucosal homeostasis. Further characterization of these pathways is warranted.

The presence of an intact and functioning intestinal mucosa is essential for health and survival. In the small intestine, maintenance of the epithelium relies upon the complex processes involved in control of mucosal homeostasis. Our current understanding of this phenomenon begins in the crypt-base stem cell niche which gives rise to all epithelial cell types [1,2]. Evidence for the role of the enteric nervous system (ENS) in crypt cell proliferation has existed for some time [3–5], while evidence for the role of the cholinergic system has been a more recent development, particularly in relation to cancer [6–8]. Previous data from our lab have suggested a role for cholinergic signaling in mucosal homeostasis using scopolamine to reverse the mucosal growth and increased crypt cell proliferation seen in the setting of enhanced serotonin signaling [9]. Other investigators have proposed a role of cholinergic neurons in control of homeostasis, and furthermore demonstrated the presence of muscarinic acetylcholine receptors (mAChRs) on the intestinal mucosa [10]. mAChRs belong to the G-protein-coupled receptor family and consist of five known subtypes, M1-M5. They can be grouped based upon the downstream effects of the particular G-protein subunit activated, with M1, M3, and M5 coupled to the $G_{q/11}$ subunit which activates phospholipase C, while M2 and M4 are coupled to $G_{i/o}$ which inhibits adenylate cyclase [11]. We hypothesized that mice with altered muscarinic receptor expression would exhibit differences in mucosal morphometric and proliferative parameters. We aimed to use mutant mouse lines deficient in each of the five mAChRs to demonstrate these differences and provide evidence for the role of muscarinic signaling in intestinal mucosal homeostasis in a murine model.

1. Materials and methods

1.1. Animals

Mutant mouse lines specifically deficient in each of the five mAChR subtypes (M1KO-M5KO) bred on a C57Bl/6 background were obtained from Taconic Biosciences. Wild-type (WT) C57Bl/6 mice were bred and housed together with the mAChR knock-out mice in a pathogen-free environment with 12-h light/dark cycle and food/water ad libitum. All mice used for experiments were adult males between the ages of 12 and 18 weeks. Animal protocols were approved by Yale University's Institutional Animal Care and Use Committee.

1.2. Histology and immunohistochemisty

Following CO_2 asphyxiation, 2 cm segments from the distal ileum were procured from animals in each experimental group and fixed in

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10% neutral buffered formalin overnight at room temperature. These were then paraffin embedded and histologic sections created for H&E and immunohistochemical staining. Standard protocols were used for both H&E and chromogenic stains. For Ki67 staining, rabbit polyclonal primary antibody against Ki67 at 1:250 dilution (Thermo Fisher Scientific, Waltham, MA) was used with anti-rabbit HRP secondary antibody at 1:500 dilution (Abcam, Cambridge, MA). Chromogen was developed using DAB substrate (Thermo) according to manufacturer protocol and counterstained with hematoxylin.

1.3. Morphometric parameters

Sections were imaged using standard brightfield microscopy (AxioScope, Carl Zeiss, Oberkochen, Germany) at 200–400×. H&E sections were analyzed using ImageJ software (NIH, Bethesda, MD) to determine villus height (VH) and crypt depth (CD). At least 30 villi and crypts were measured per group. Villi were chosen for measurement when intact from crypt–villus junction to crypt–villus junction with a visible central lacteal. Crypts were measured when intact from crypt–villus junction to crypt–villus junction and with at least partial visualization of adjacent villi.

1.4. Crypt proliferation index

Ki67-stained slides were used to calculate crypt proliferation index (CPI) using images obtained as above. CPI was calculated by counting the number of Ki67 positive cells (cells within the crypt positive for brown DAB chromogen), dividing by the total number of crypt cells and converting this number to a percentage. Crypts were counted if a single epithelial cell layer was present from crypt–villus junction to crypt–villus junction, and at least a portion of the adjacent villi were present. At least 5 crypts per group were evaluated, and used to calculate the mean.

1.5. Statistical analysis

Statistics were performed using Prism Software (GraphPad, San Diego, CA). Groups were compared using Tukey's multiple comparisons test, with significance assumed when $p < 0.05$.

2. Results

Distal ileal segments from mice deficient in mAChRs showed significant differences in nearly all measured parameters (Table 1 and Fig. 1). The ileum from mice deficient in specific muscarinic receptor subtypes (M1KO-M5KO) showed consistently increased measures for CD when compared to WT, while results for VH were more variable. M3KO and M5KO had significantly increased, M2KO significantly decreased, and M1KO and M4KO had no differences in VH compared with WT. For CPI, all subtypes except M4KO had significant increases compared to WT (Table 1, Fig. 1C). In general, CPI results seemed to correlate with those for CD, with M3KO and M5KO having the largest increases compared to WT (Table 1). When mAChR groups were compared amongst each other significant differences and several potential trends were observed (Fig. 1). First, no differences were observed between M1KO and M4KO or between M3KO and M5KO groups for any of the measured parameters. Furthermore, M3KO and M5KO groups had the greatest increases from WT for all three parameters. Lastly, M2KO, M3KO and M5KO had significant changes from WT in all three parameters. Representative images of H&E and Ki67-stained slides (Fig. 2) demonstrate the observed differences in morphometric and proliferative parameters.

Table 1
Morphometric and proliferative measures for WT and mAChR-knockout groups.

	Villus height (μm)	Crypt depth (μm)	Crypt Proliferation Index (%)
Wild type (WT)	205 ± 4	46 ± 1	35 ± 3
M1KO	208 ± 4	58 ± 2*	52 ± 3*
M2KO	184 ± 3*	71 ± 3*	56 ± 2*
M3KO	223 ± 4*	82 ± 2*	57 ± 1*
M4KO	211 ± 6	67 ± 2*	43 ± 4
M5KO	229 ± 4*	81 ± 2*	58 ± 1*

* $P < 0.05$ when compared to WT.

3. Discussion

The contribution of the various subtypes of muscarinic acetylcholine receptors (mAChRs) to intestinal mucosal homeostasis in mice was investigated. Mutant mouse lines specifically deficient for each receptor subtype (M1KO-M5KO) were examined for differences in morphometric and proliferative parameters in the small intestine. There has been an increase in interest in the cholinergic signaling system and its role in cancer biology [6,7,12,13], however very little is known about this system as it relates to homeostasis of the small intestinal mucosa. Recent evidence has implicated the cholinergic system in protection from the apoptosis induced by cellular damage and stress [14] and inhibition of epithelial barrier dysfunction accompanying inflammation [15]. Additionally, various reports have identified the muscarinic receptors throughout the murine gastrointestinal tract [16], but the function of these receptors in the small intestine has not been well studied. Characterization of these mechanisms would be an important step in the process toward eventual therapeutic manipulation of these pathways to induce the growth of functioning small bowel mucosa, which has potential benefits for patients suffering from intestinal insufficiency such as those with short bowel syndrome. Previous data from our lab suggest a role for muscarinic signaling in serotonin-mediated intestinal mucosal growth [9], and given that serotonergic neurons are not known to project to the mucosa, the link between the serotonin signaling system and the epithelium may be the muscarinic acetylcholine signaling system. Furthermore, if the cholinergic system serves as the final link to the epithelium in the setting of enhanced serotonin signaling, it may be the final common pathway for a number of signaling systems in the ENS, increasing its appeal as a therapeutic target. We thus hypothesized that genetic knockout of the mAChR subtypes would provide insight into the role of cholinergic signaling in control of homeostasis in the intestinal epithelium.

Examination of distal ileal segments from the five receptor subtypes revealed significant differences in most of the measured parameters suggesting that muscarinic signaling plays a role in epithelial homeostasis. Interestingly, CD and CPI measurements seemed to correlate with each other, while VH measurements were somewhat unexpected. For CD, all mAChR subtypes showed significant increases compared to WT (Table 1 and Fig. 1B), while all groups except M4KO showed significantly increased CPI (Table 1 and Fig. 1C). Examination of the pattern of results reveals a potential correlation with the G-protein subunits with which the mAChR subtypes are known to be coupled [11]. M1, M3, and M5 mAChRs couple to the $G_{q/11}$ subunit which activates phospholipase C, while M2 and M4 are coupled to the $G_{i/o}$ subunit which inhibits adenylate cyclase. The M3KO and M5KO groups had the largest increases for all parameters. These results suggest activation of the $G_{q/11}$ cascade in this setting results in inhibition of proliferation, such that knockout results in disinhibition. The results for CD and CPI in the M2KO and M4KO groups, however, do not appear to correlate with this proposed model. Activation of the inhibitory $G_{i/o}$ pathway would not be expected to result in increased crypt growth and proliferation, unless these groups of receptors are acting at different points in the pathway. For VH, significant differences were seen when compared to WT, but in an unclear pattern (Table 1 and Fig. 1A). M1KO and M4KO both appeared to have no effect on VH, while M3KO and M5KO appeared to have a stimulatory effect. Interestingly, the M2KO group had an inhibitory effect on VH. This pattern of results could also be partially explained by the known associations of the mAChRs to G-protein subunits. With