The role of fibroblast-like cells in purinergic neuromuscular transmission in colitis

Experimental Aims
This study was designed to test the hypothesis that the colitis-induced deficit in purinergic neuromuscular transmission that has previously been identified by the Mawe lab involves a disruption in the population of fibroblast-like cells (FLC) of colonic muscularis. Our previous publication demonstrated that the purinergic component of the inhibitory junction potential (IJP), the signal responsible for relaxation below a stimulus during peristalsis, could be a contributing factor in impaired intestinal motility. A possible mechanism by which the purinergic component of IJP is attenuated involves FLCs, a type of interstitial cell, as these cells are thought to mediate purinergic neuromuscular transmission in the gut. Examining the density and distribution of FLCs in inflamed colonic tissue is a first step in understanding the possible contribution of these cells to dysmotility in inflammatory bowel disease (IBD).

Approach
The specific aims of the study were to determine the density and distribution of FLCs in inflamed and normal colon, as well as to evaluate the proportion of FLCs having nerve fibers in close apposition in inflamed and control groups. Immunohistochemistry (IHC) was used to selectively stain FLCs in normal and inflamed colon using two complementary models of colitis, guinea pig trinitrobenzene sulfonic acid (TNBS) colitis and murine dextran sodium sulfate (DSS) colitis. The use of two animal models was important because neither is a perfect representative of IBD, but they share features of Crohn’s disease and ulcerative colitis, respectively.

Albino guinea pigs were given a TNBS enema (0.3 ml of a solution of 25 mg/ml TNBS dissolved in 30% ETOH) to induce inflammation, as previously described. On the sixth day following TNBS administration, the animals were euthanized by isoflurane overdose and exsanguination. The distal colon was removed, and macroscopic damage scores were used to assess the extent and severity of colitis in the explanted colon. Scores are determined by parameters such as intestinal wall thickness, extent of ulceration, fecal blood, and adhesions (Fig. 1). TNBS guinea pigs in the present experiment had a mean score of 5 (range 3 to 7). To induce colitis in the murine model, male BALB/c mice were given drinking water containing dissolved DSS at a concentration of 4% (wt./vol.)2. The mice were euthanized in the manner described above after 5 days of drinking the DSS-containing water, followed by 2 days drinking tap water. Macroscopic damage scores were used to assess the extent and severity of colitis in mouse colon, with measured parameters similar to those used in guinea pig. DSS mice in the present experiment had a mean score of 2.67 (range 1.67 to 3.67) (Fig. 2). Colons from naïve guinea pigs and mice were used as controls. The methods presented above are currently in use in the Mawe laboratory, and were approved by the University of Vermont Animal Care and Use Committee (IACUC Protocol 12-037).

In preparation for IHC, explanted colons were stretched in iced Krebs with 0.1 mL nifedipine to facilitate muscle relaxation. The colons were then fixed overnight in 4% paraformaldehyde. Following fixation, the mucosal surface was examined with a dissecting microscope, photographs were taken (Fig. 1), and maps of inflamed regions were drawn. The mucosa and submucosa were then removed, and full thickness specimens with intact circular muscle, myenteric plexus, and longitudinal muscle were taken from sites
of inflammation, as well as from a second site approximately 1 cm away. Specimens were taken from control colons at corresponding sites. Colonic specimens were incubated in 0.1% bovine serum antigen (BSA), 4% normal horse or normal goat serum (for mouse and guinea pig, respectively) in phosphate buffer solution (PBS)/0.5% Triton-X for 2 hours to reduce nonspecific binding. The specimens were then incubated for 48 hours at room temperature in primary antibody. For guinea pig specimens, anti-rabbit PDGFR-α (1:200) was used to stain for FLCs and anti-mouse PGP9.5 (1:800) was used to stain for neurons. For mouse specimens, anti-goat PDGFR-α was used to stain for FLCs and anti-rabbit NOS (1:500) was used to stain for inhibitory neurons. Following treatment with primary antibodies, the specimens were washed in PBS and incubated for 2.5 hours at room temperature in secondary antibodies. Finally, the specimens were washed in PBS, mounted on glass slides using Citifluor, and photographed using an Optronics MagnaFire CCD camera attached to the Olympus AX70 microscope.

Results
While the Mawe laboratory has extensive experience with immunohistochemical staining of intestinal preparations, this is the first time our group has used antibodies directed against PDGFR-α, and immunostaining for PDGFR-α had not been previously published in the guinea pig gut. Therefore, initial experiments were conducted with normal mouse and guinea pig colonic tissue to determine the optimal primary and secondary antibody dilutions to use in order to obtain high quality immunostaining that would allow for assessment of FLC densities and FLC-nerve associations. Figures 3A and B illustrate double label immunostaining for PDGFR-α plus a neuronal marker in mouse and guinea pig, respectively. Once the immunostaining conditions were optimized, I have been conducting immunostaining with samples from naïve and inflamed mice and guinea pigs. Based on a qualitative evaluation of the preparations, it appears that the density of FLCs is decreased in the inflamed conditions in both murine and guinea pig colitis (see Fig. 3). The next step will be to use unbiased morphometry techniques to evaluate the density and distribution of antibody labeled FLCs in normal colon, inflamed colon, and normal appearing regions adjacent to inflamed regions. Furthermore, the proportions of FLCs in close apposition with nerve fibers in control and inflamed preparations will be determined.

Challenges
Previous studies have stained for FLCs in mice, so some information was available on antibody selection and dilutions. In guinea pigs, however, there was very little information on staining for FLCs, so it required several trials with varying antibodies, dilutions, and incubation times to arrive at a protocol that allowed for the evaluation of these cells.

Future Directions
In a recent study from the Mawe lab, Roberts et al. demonstrated that oxidative stress contributes to the attenuation of purinergic signaling at the neuromuscular junction in inflamed colon, and that treatment with a free radical scavenger improved IJPs and colonic motility². If significant differences in FLC density and/or FLC-nerve association are observed in the present study, an examination of the effects of a free radical scavenger on FLCs may help to further elucidate the mechanisms responsible for neuroplastic changes that occur in colitis.

References

Figure 1: TNBS-induced ulceration in guinea pig colon.

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<th>Weight Loss</th>
<th>Stool Consistency</th>
<th>Fecal Blood</th>
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Figure 2: Macroscopic damage scoring system in murine model. Weight loss, stool consistency, and fecal blood are used to determine the disease activity index (DAI).
Figure 3A and B: Micrographs of whole mount preparations of mouse and guinea pig colon immunostained for PDGFR-α and neuronal markers. Note that the density of PDGFR-α–positive cells appears to be lower in the inflamed preparations.