Serine Proteases as Potential Markers for Diagnosis and Treatment of Ulcerative Colitis

Patrick Ma[1]; Adelpha Chan[1]; Zhenze Jiang[1]; Anthony J. O'Donoghue[1], Ph.D; Dennis W. Wolan[2], Ph.D

[1]*Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093*

[2]*The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037*

Overview

Purpose: To characterize proteolytic activities in ulcerative colitis (UC) samples and identify potential protease targets for diagnosis and treatment of UC

Methods: Perform fluorescent assays to assess protease activities of colonic and fecal samples obtained from colitis mouse models

Results: Significant differences in serine protease activities were identified between colitis and non-colitis mice

Introduction

Ulcerative colitis (UC), one of the two major branches of inflammatory bowel disease (IBD), has a costly, imprecise diagnosis[1]. Diagnosis often requires blood and fecal tests, in addition to colonoscopies and histologies[2]. Caused by a faulty immune system reacting inappropriately to surrounding stimuli, IBD results in inflammation and ulcer development in the colon and rectum, leading to rectal tenesmus, bloody defecation, diarrhea, and an increased risk of colon cancer[3]. Immune system suppressors and anti-inflammatory drugs minimize inflammation and decrease flare-up frequency, albeit often poorly[4].

To better understand ulcerative colitis, we used recombination activating gene (Rag) knockout mice containing a genetic mutation that prevents the development of T lymphocytes (T cells). Rag mice injected with T cells have been previously demonstrated to exhibit a chronic colitis phenotype similar to that of humans[5]. In this study, we extracted protein from colonic and fecal samples of such mice and assayed with sixteen fluorescent substrates. We found significant differences in serine protease activities between colitis and control mice. Previous studies have suggested that inhibiting certain proteases can decrease the symptoms of IBD[6,7]. These serine proteases, if further investigated, could potentially prove to be biomarkers for a simpler diagnosis and drug targets for more effective treatment.

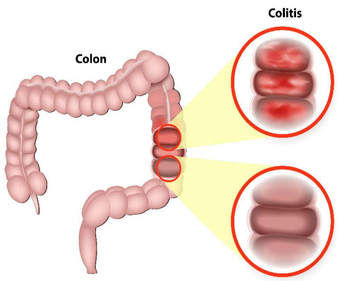


Figure 1. Colitis causes inflammation in the colon

Methods

- **Mice Models**:

Mice colonic and fecal samples were provided by Dr. Dennis Wolan of The Scripps Research Institute. Wild type mice are a control representing healthy patients. Rag -/- mice are a control representing a lack of immune system activity. Rag -/- with T cell transfer mice represent an over-activity of the immune system as seen in ulcerative colitis patients.

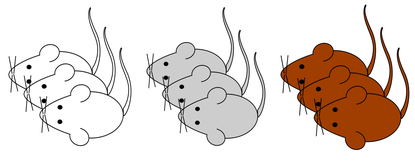


Figure 2a. Three wild-type mice (white), three RAG -/- control mice (gray), three RAG -/- + T cell mice (brown)

- **Protein extraction:**

Fecal samples collected pre-sacrifice and colonic samples collected post-sacrifice eleven days later were obtained from three WT, three Rag -/- control, and three Rag -/- + T cell mice. Samples were suspended in PBS (Thermo), homogenized, vortexed, and centrifuged for 13.5k xg at 4 °C. The resulting supernatant was collected and used for fluorescent assays.

- **Fluorescent Assay:**

5 µg/mL of the protein samples was mixed with 10 µM fluorescent substrate in assay buffer (PBS, 0.01% Tween). Fluorescence was read at excitation and emission wavelengths of 360 nm and 460 nm respectively, on a Synergy HTX Multi-Mode Microplate Reader (BioTek). For inhibition assays, 1mM AEBSF inhibitor was incubated with the samples for 15 minutes at room temperature prior to the addition of the fluorescent substrate.

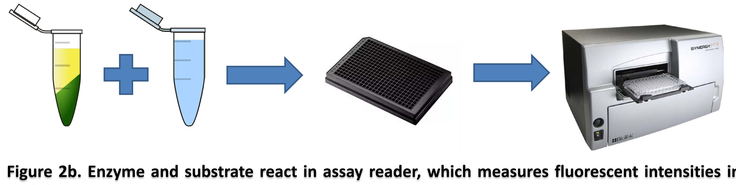
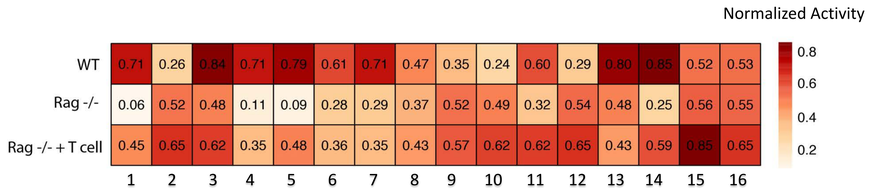


Figure 2b. Enzyme and substrate react in assay reader, which measures fluorescent intensities in real-time.

Results



1. Bz-Phe-Val-Arg-AMC
2. Arg-AMC
3. Gly-Arg-AMC
4. Glutaryl-Gly-Arg-AMC
5. Val-Leu-Thr-Lys-AMC
6. Leu-Leu-Val-Tyr-AMC
7. Pro-Asp-Phe-Tyr-ACC
8. Tyr-Val-Ser-Ala-ACC
9. Ala-AMC
10. Nle-AMC
11. G-NLE
12. Ile-Ble-Gln-Thr-ACC
13. MeOSuc-Ala-Ala-Pro-Val-ACC
14. Suc-Ala-Ala-Pro-Phe-ACC
15. Glu-Trp-His-Trp-ACC
16. Ac-Ala-Ile-Lys-ACC

Figure 3a. Heatmap showing fluorescent substrates screened with the colonic samples. The numbers in each box are the average normalized activity of three mice of the same type. WT: Wild-type; Rag -/-: Rag knockout; Rag -/- + T cell: Rag knockout with T cell transfer.

In this study we saw higher trypsin-type activity (as assessed by substrates 1, 4, 5) and chymotrypsin-type activity (as assessed by 14) when assayed with Rag -/- + T cell mice samples, which are animal models of UC, compared to the Rag -/- control mice samples. We propose that some serine proteases become more active at the onset of colitis. To confirm our hypothesis, we then selected several substrates to perform inhibition assays using AEBSF, a known serine protease inhibitor.

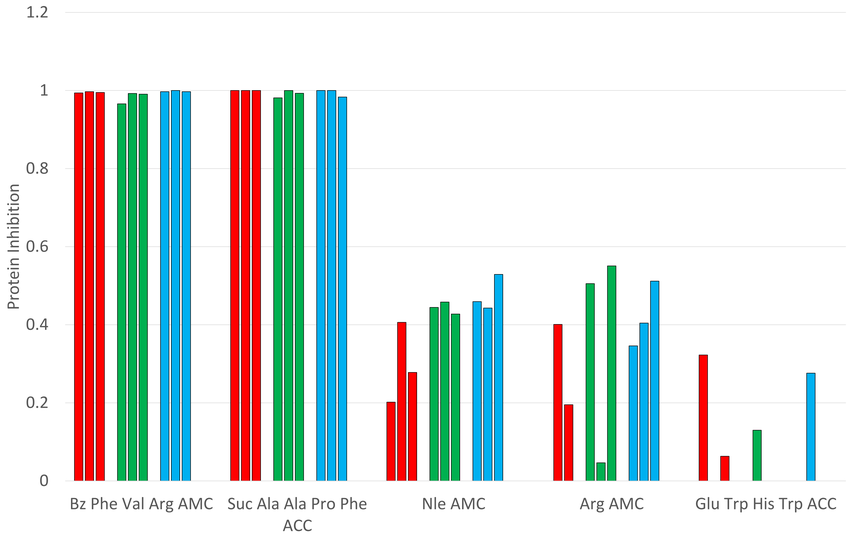


Figure 3b. Results of AEBSF inhibition assays with colonic samples. Protease activity is largely inhibited when assayed with substrate 1 (trypsin-type) and substrate 14 (chymotrypsin-type), partially inhibited with substrate 2 and 10 (aminopepsidase), and not inhibited with substrate 15 (endopepsidase). Red: WT, green: Rag -/- , blue: Rag -/- + T cell.

High inhibition of substrates 1 and 14 suggests a high presence of serine protease. This confirms our hypothesis that the activity difference between Rag -/- control and Rag -/- + T cell mice when assayed with substrate 1 and 14 is due to serine protease activity.

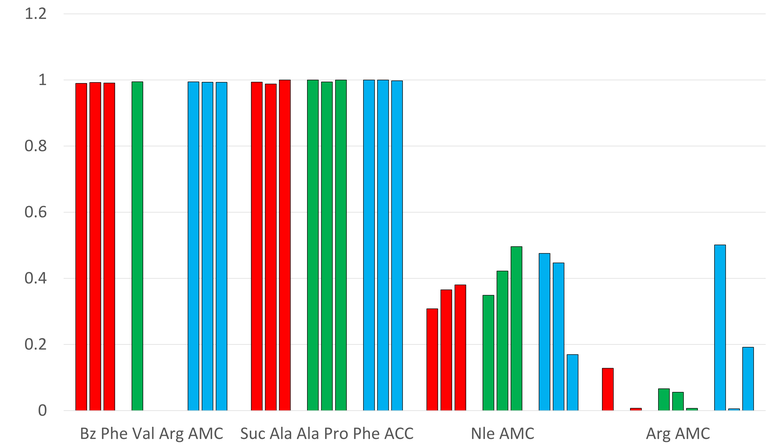


Figure 3c. Results of AEBSF inhibition assays with fecal samples. Color scheme same as figure 3b. The proteolytic activities of fecal samples were similar to that of colonic samples.

Conclusions

- Colonic and fecal samples of WT, Rag -/- and Rag -/- + T cell mice were used to study the extracellular protease activities of ulcerative colitis.
- This study demonstrates the association of certain protease activities with the onset of colitis by assaying with a panel of fluorescent substrates. The activities were identified to be derived from serine proteases with inhibition assays.
- There are small differences in serine protease activities between colonic and fecal samples, demonstrating that passage through the rectum and an eleven day development of colitis have little to no effect on protease activity.
- The findings of this study could contribute to the identification of serine proteases as biomarkers for effective point-of-care diagnostic techniques and as potential drug targets for the treatment of UC.

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