Identification of proteases as potential targets for the treatment of ulcerative colitis

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Abstract

The purpose of this experiment was to examine protease activity among various substrates in order to identify distinctive biomarkers in Rag -/- + T cell mice, representative of ulcerative colitis patients. Fecal and colonic samples were collected from Wild type, Rag -/-, and Rag -/- + T cell mice. Samples were assayed with a panel of fluorescent substrates +/- AEBSF inhibitor. The study showed elevated serine protease activity in the Rag -/- + T cell model in comparison to the Rag -/- control. The identification of this distinct proteolytic activities can be further investigated, possibly leading to the discovery of protease targets that could be used for the development of therapeutics suppressing the inflammation caused by overactive T cells, which enhance the symptoms of ulcerative colitis.

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD), the primary constituents of inflammatory bowel disease (IBD), are chronic inflammatory disorders of the gastrointestinal (GI) tract which affect ~1 million individuals, with ~30,000 new cases reported annually in US[1-3]. Patients of UC and CD suffer from severe diarrhea, abdominal pain, weight loss, rectal bleeding and are at risks of toxic megacolon, perforated colon and even colon cancer. Diagnosis of IBD involves blood, fecal tests, colonoscopy and histology. As IBD is the result of an overactive immune system and T cells that attack patient's own body, causing inflammation, common treatment methods include anti-inflammatory drugs and immune system suppressors. Though there are palliative drug and surgical treatment options for this chronic disease, there is currently no cure.

Previous studies have shown that dysregulated proteolytic activities are associated with IBD development. There is evidence showing the recovery of specific proteolytic homeostasis and the inhibition of certain proteases can ameliorate the inflammatory symptoms[4-5]. In this study, we collected colonic and fecal samples from Rag-/- mice with T cell transfer[6] and assayed samples with a panel of fluorescent substrates. The results showed distinct serine protease activities between the UC mice model (Rag -/- + T cell) and the control (Rag -/-). These findings could potentially lead to the identification of serine proteases that are involved in the development of IBD. The proteases could be a possible biomarkers for accurate diagnosis and drug targets for the treatment of diseases.

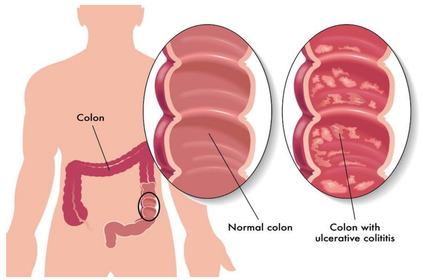

Fig 1. T cells attacking one's own colon results in inflammation and ulcers, causing colitis[7]

Methods

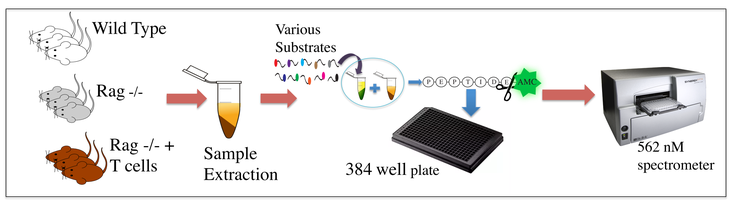

Fig 2. Workflow of the Experiment. The colonic and fecal samples of wild type, Rag -/-, and Rag -/- with T cell transfer mice were used in experimentation. Proteins were extracted from the samples and assayed with 15 various fluorescent substrates for proteolytic activities.

- **Mice Models**: Mice colonic and fecal samples from Dennis Wolan's laboratory at Scripps Research Institute. Wild type mice acted as a control representing healthy patients. Rag -/- mice are a control representing a lack of immune system activity. Rag -/- with T cell transfer to observe the activity representative of over-activity seen in ulcerative colitis patients.
- **Protein Extraction:** Both colonic and fecal samples prepared using identical methods. 500 µL of PBS (Thermo) was added into each sample. Pellets were broken up within the buffer solution using a pestle homogenizer, vortexed and centrifuged for 10 min at 13.5 k x g at 4°C. Supernatants was aliquoted and stored at -80°C for fluorescent assays. Protein concentration was determined using BCA assay with bovine serum albumin as a protein standard.

Fluorescent Assay: 5 µg/mL of the protein samples were assayed with 10 µM fluorescent substrate in PBS (Thermo)+0.01% Tween buffer. Fluorescence was read at Ex/Em= 360/460 nm on a BioTek Synergy HTX Multi-Mode Microplate Reader. For inhibition assays, Assays were performed in the presence and absence of 1mM AEBSF.

Results for NE/SA Assays

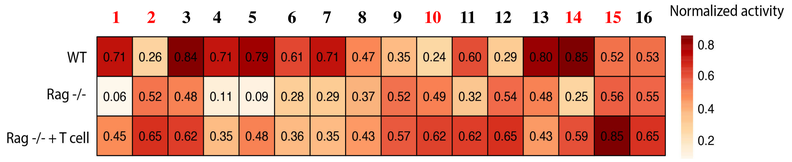

Figure 3. Heat map showing average protease activity of the three mice of each type screened with 16 fluorescent substrates in colonic samples.

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

Table 1. Fluorescent substrates used in assays

It was observed that substrates 1, 4, and 5 exhibited much activity. In addition, single amino acid substrates exhibit similar activity between the control (Rag -/-) and treated mice (Rag -/- + T cell). Results show that substrates 1, 4, 5, 14 and 15 show noticeable differences in protein activity between the three types of mice, especially Rag -/- and Tag -/- + T cell mice.

Red numbered substrates were used for AEBSF inhibition assays. These substrates were chosen for the inhibition assay as they contain peptide sequences favored by serine proteases that are known contain major differences in activity between Rag -/- and Rag -/- + T cell mice.

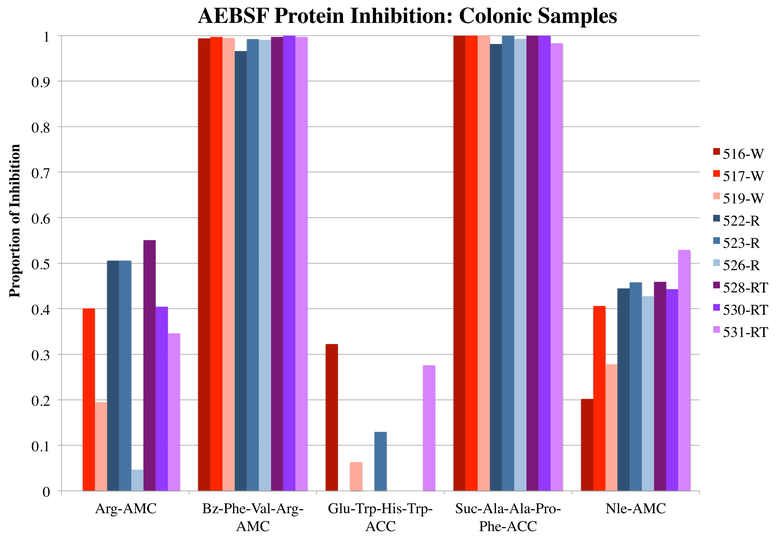

Figure 4. Results of AEBSF inhibition assays with colonic samples. Results show that substrates Bz-Phe-Val-Arg-AMC and Suc-Ala-Ala-Pro-Phe-ACC were almost completely inhibited by the AEBSF inhibitor in all mice samples.

As AEBSF is a serine protease inhibitor, substrates Bz-Phe-Val-Arg-AMC and Suc-Ala-Ala-Pro-Phe-ACC must be substrates exhibiting serine protein actvity. However, substrates Arg-AMC and Nle-AMC are only partially inhibited. As these substrates are only partially inhibited, they must contain some serine proteases that can be inhibited AEBSF but also other types of proteases, such as aminopeptidases and carboxypeptidases.

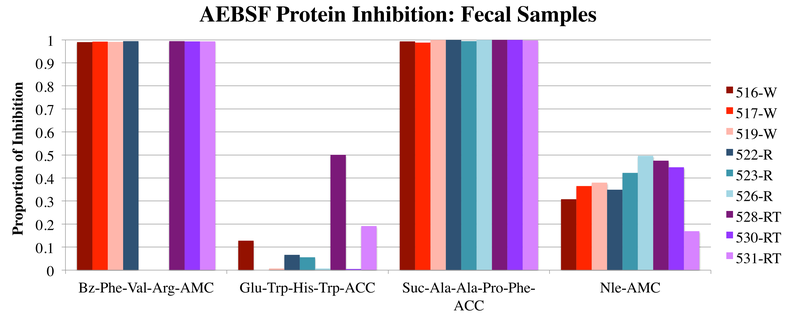

Figure 4. Results of AEBSF inhibition assays with fecal samples. Results show that substrates Bz-Phe-Val-Arg-AMC and Suc-Ala-Ala-Pro-Phe-ACC were almost completely inhibited by the AEBSF inhibitor in all mice samples.

In comparison to the inhibition assays done with colonic samples, substrates exhibiting only serine protease activity and partially inhibited substrates are similar, showcasing that the passage through rectum probably does not change the protease activity of the sample.

Conclusions

According to the results of the assays of the 16 various fluorescent substrates, as showcased in the heat map, it can be seen that in most substrates, there is a noticeable difference in protease activity among wild type, rag -/-, and rag -/- + T cell mice. Specifically, substrate 1, 4, 5, 14 and 15 show extreme differences between the colitis mice and the control. With the AEBSF inhibitor assays, protease activity in substrates 1 and 14 were completely inhibited.

The purpose of this study is to identify whether there are proteolytic activities specific to colitis mice. Though AEBSF only partially inhibited the activity with substrate 2 and 15, the fact that AEBSF was able to completely inhibit the activity of substrate 1 and 14, posing promising results that certain serine proteases could potentially act as key players in the development of UC. These proteases could be further enriched, extracted and characterized to understand their biological functions and their relevance to UC, which could possibly be used as new target drugs for therapeutics that inhibit overactivity in T cells for the treatment of symptoms of inflammation and ulcers seen in colitis patients.

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Acknowledgements

First, we would like to thank Dr. Anthony O'Donoghue and Dr. Dennis Wolan for providing mentorship and aid throughout our time at the Skaggs School of Pharmacy and Pharmaceutical Sciences. We would also like to thank the rest of the O'Donoghue lab, specifically Sandeep Adam and Steven Wang for taking on the role of a mentor during the second half of our stay .Lastly, we would like to thank Dr. Elizabeth Komives for all her time and efforts that made this research experience possible.