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Acute Graft versus Host Disease: Studies in Histology And the Role of Tissue Repair and Regeneration

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Abstract:
Acute graft-versus-host disease (aGvHD) occurs when donor T-lymphocytes attack host tissues; the disease can be life threatening. Recently, elevated circulating Follistatin (FS) was described in those experiencing aGvHD; higher concentrations of this factor were associated with poorer survival and greater risk for the disease. The mechanism behind these increased FS levels is unknown. As such, it is essential that we further study the tissue-specific expression of FS. As aGvHD greatly affects the intestinal tract, we hypothesize that studying FS expression in the intestinal tract will provide a better understanding of the role of FS in aGvHD. This study can be completed by a multi-step process. This paper focuses on the first step: the histological differences in the intestine of individuals with or without aGvHD. We did this by performing Hematoxylin and Eosin Stains on aGvHD affected versus control intestinal mouse tissue and compared the tissue histology of the two groups. We found that aGvHD affected tissues appeared to have disorganized villi, in which the inter-villi distance varied significantly more than control tissues, in which the villi appeared to be more organized. This indicates that aGvHD causes an abnormality in organizational proteins that give rise to villi. This is of importance as an organizational abnormality may be the reason for the symptoms of aGvHD such as digestion problems and diarrhea. Now that our model is established and shows consistent differences in histology, tissues can be stained for FS, and FS signaling pathways can be interrogated using molecular arrays.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is one of the modalities to cure many hematologic malignancies like leukemia, lymphomas, and non-malignant conditions like aplastic anemia and sickle cell anemia. A major complication of this therapy is graft-versus host-disease (GVHD). Acute graft-versus-host disease (aGvHD) occurs when donor T-lymphocytes attack host tissues. This complication arises in approximately 50% of hematopoietic stem cell transplant recipients, impacting about 5500 patients annually (Bhattacharyya et al., 2010). About half of these immunocompromised patients do not successfully respond to first line treatment with steroids, and many develop severe infections. As a result, aGvHD can be life threatening (Chen et al., 2014). This disease primarily affects the skin, liver, and gastrointestinal tract (Jacobsohn et al., 2010). Identification of novel novel treatment approaches that can alleviate inflammation, prevent infection, and promote healing is critical for improving patient outcomes.

Using mouse models, it has been demonstrated that inflammatory neovascularization accompanies aGvHD. Thus, many studies to understand the angiogenic milieu of aGvHD have been performed to identify biomarkers associated with development of aGvHD. Our group recently demonstrated that an angiogenic factor follistatin (FS) is associated with aGVHD. We found that higher pre-transplant plasma FS concentrations in both donors and recipients are associated with increased incidence of aGvHD, and in increased mortality risk in allogeneic HSCT recipients (Turcotte et al., 2017). However, the source of circulating FS and biological mechanisms behind these increased FS levels has yet to be determined.

FS, a glycoprotein that primarily functions

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functions in the binding and neutralization of transforming growth factor-beta (TGF-B) superfamily members, has increasingly appreciated roles in tissue repair and inflammation. FS was first described as a follicle-stimulating hormone suppressing substance in ovarian follicular fluid 30 years ago (Ying et al., 1987), although more recent studies have shown its relevance in many clinical settings. In addition to supporting repair processes, FS is an angiogenic factor and can also promote muscle growth via inhibiting myostatin and promote energy metabolism by inducing adipocyte differentiation (Rodino-Klapac et al., 2009; Braga 2014). While the tissue-specific effects of FS in inflamed or damaged tissue are difficult to study in humans, several studies have identified elevated plasma FS levels as a pathologic biomarker in various conditions including liver disease, chronic renal failure, advanced solid cancer, septicemia, hyperthyroidism, and obesity (Flanagan et al., 2009; Rinnove et al., 2013 Tseng et al., 2016). Prior research has shown that the inhibition of activin-A by FS allows for tissue regeneration when tissue is lost due to injury in planarians (Galbraith et al., 2013).

FS is also well known for its roles in inflammation. It inhibits activin-A a protein which plays a critical role in the inflammatory cascade response. (Inouye et al., 1991a; Nakamura et al., 1990; Sugino et al., 1993). Recently, elevated circulating FS was described in recipients of allogeneic HSCT experiencing aGvHD, where higher concentrations were associated with poorer survival (Holtan et al., 2015; Turcotte et al., 2017). Higher plasma levels of FS in HSCT donors were associated with a greater risk of aGvHD in HSCT recipients (Turcotte et al., 2017). Given the association of FS and clinical outcomes related to aGvHD, further study into the tissue-specific expression and effects of FS is warranted.

FS is expressed in nearly all tissues of mammals including skeletal muscle, pituitary gland and brain, and endothelial cells, and its expression depends on cellular proliferation and/or turnover. As aGvHD greatly affects the intestinal tract, we hypothesize that studying the FS expression in intestinal tract will provide a better understanding of the role of FS in aGvHD. This can be achieved in a multi-step process. The first step towards this is to study the histological differences in the intestine of individuals with or without aGvHD. In this project, our group will study the expression of FS in the small intestine of aGvHD mice and control mice to establish a model for future experimentation. In a subsequent study, our group will also analyze the biopsies of human aGVHD for FS.

Methods

Mice

C57 BL/6 mice were purchased from the National Institutes of Health. B10. BR/SgSnJ (B10BR) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These B10BR mice have MHC alleles which greatly differ from C57 BL/6 mice (i.e., major MHC mismatch transplant). All mice were bred and kept in a pathogen-free facility in microisoslater cages in which they were monitored daily according to IRB protocol. When the B10. Br recipient mice were mature (8-12 weeks of age), they were conditioned with 120 mg/kg of cyclophosphamide for two days. On the second day, the recipients were also irradiated with 8.0 Gy of total body irradiation. The purposes of this conditioning regimen with chemotherapy and radiation is to ablate the host bone marrow and sufficiently immunosuppress them such as the mismatched donor cells could engraft and not be rejects. After conditioning, these mice were infused with bone marrow from the C57 BL/6 mice (BM) (20×106). This marrow was depleted of T cells using anti-Thy-1.2 plus rabbit complement treatment (Blazar et al., 1999). To induce GVHD in a subset of the mice, donor splenocytes (a rich source of T-cells) were also infused after the bone marrow transplant. Mice were weighed twice weekly and monitored daily for survival and clinical evidence of GVHD (ruffled fur, cachexia, alopecia, diarrhea) (Blazar et al., 2003). At 49 days post-HSCT, 4 aGvHD mice (MHC mismatch T-cell depleted donor marrow plus splenocytes) and 4 control mice (MHC mismatch T-cell depleted donor marrow and no splenocytes), were euthanized and the small intestines were removed for histopathologic analysis.

Tissue sectioning and staining

The colons of all 8 mice were snap frozen in liquid nitrogen and stored at -80°C, in separate blocks. Frozen sections were cut 5 μm thick and fixed for 5 min in acetone. To determine the histological differences between aGvHD and control samples, Hematoxylin and Eosin (H&E) staining was performed. The sections were exposed to hematoxylin for five minutes, washed, and then they were counterstained with Eosin.

Microscopy

Multiple brightfield images were captured at 20x magnification using a Lumos Microscope for each sample. Images that contained multiple villi were then selected for analysis. A quantitative histological analysis was performed on these samples. Specifically, average villus

villus height and the variation in distance between villi were quantified for all samples (Figure 1). These data were then tested for significance in R-Studio.

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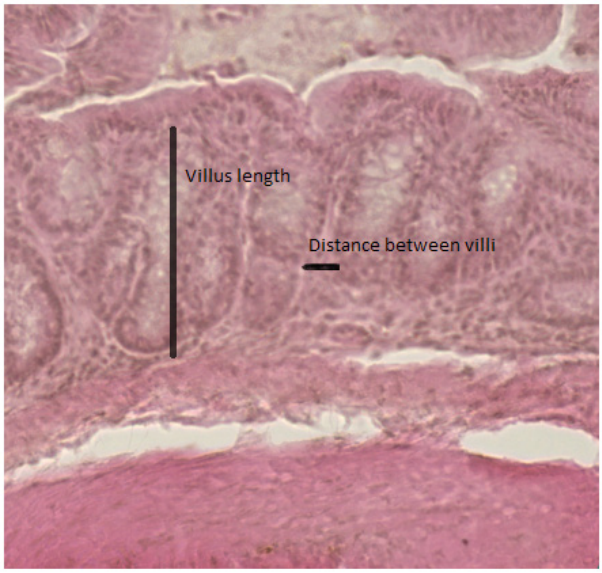


Figure 1. Vertical line demonstrates the villi length that is measured and horizontal line demonstrates the inter-villous distance

Statistical analysis

A non-heteroscedastic two-sample t-test was used to determine whether villus height was significantly different between aGvHD and control samples, and a F-test for variance (R-Studio) was used to determine whether the variation in distance between villi was significant between the two samples.

Results

Analysis of histology demonstrated that there were both qualitative and quantitative changes between the aGvHD and control mice which are summarized next.

Qualitative changes

Qualitative evidence shows that there are distinct differences between the aGvHD and control tissue histologically (Figures 2 and 3). Control samples have similarly sized villi- which appear to be longer than those of aGvHD samples- that are parallel to one another and a decreased frequency of apoptotic/necrotic cells. Additionally, the crypt sizes are uniform. (Figure 2). In contrast, aGvHD samples have disorganized villi, in which the villi are unequal distances from each other and are not parallel to one another (Figure 3). There is also increased incidence of necrosis, as can be seen clearly in aGvHD sample 3.

Quantitative changes

a. Villi height

The mean villi length of 4.85μm in the control tissue was on average greater than that of aGvHD samples which was 3.86um (Figure 4). However, this result was not statistically significant ($p=0.38$, Two-Sample Heteroscedastic T-test, Rstudio), possibly due to small sample size.

b. Inter-villous distance

The distance between the villi in the control tissue was almost constant with mean value of 0.03um (Figure 5). However, the distance between the villi in the aGvHD mouse varied a lot and was significantly higher at 1.18um ($p<0.01$, F-test, R-Studio).

Discussion

Despite decades of advancements in understanding the biology of aGvHD, immunosuppressive therapy consisting of either topical or systemic corticosteroids remain the first-line treatment for aGvHD (Garnett et al., 2013). This therapy is detrimental to patient health as it greatly compromises the immune system and thus can increase the risk for death due to infection. It also does not address problems with the underlying tissue destruction, the inflammatory neovascularization, or potential defects in intestinal epithelial restitution after injury. In patients who develop the most severe form of aGVHD, steroid-refractory aGvHD, the treatment paradigm has still focuses on further intensification of immunosuppression, with most patients dying of organ failure or infections and little progress made other than supportive care over the past few decades.

Instead of focusing on broad T-cell immunosuppression, our group has been exploring new potential avenues for aGvHD therapy, based upon lack of progress made with intensified immunosuppression in clinical trials and in clinical practice, to find ways to enhance tissue regenerative capacity order to circumvent the effects of this disease and improve survival. To begin this work, we need a thorough understanding of the biological changes that occur following aGvHD, focusing on host effects than on the donor T cells. Our laboratory recently found

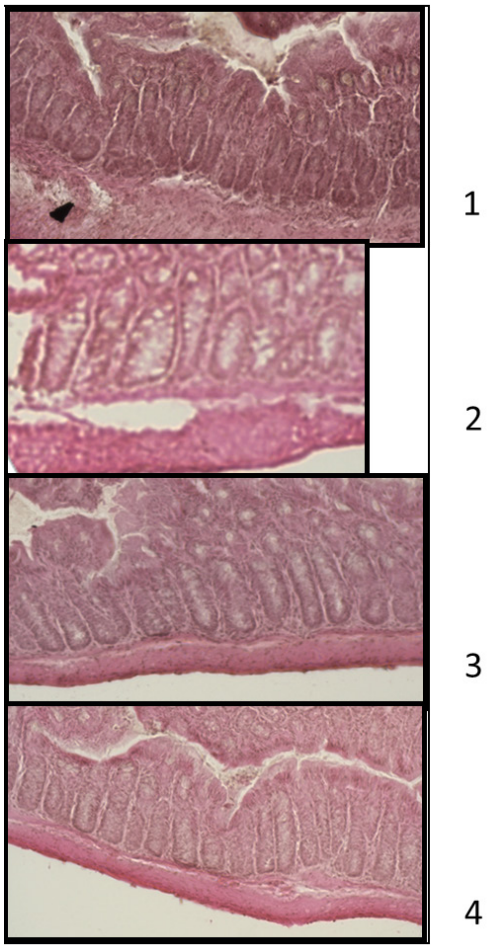


Figure 2. H&E stained intestinal tissues from four different control mice demonstrating normal architecture

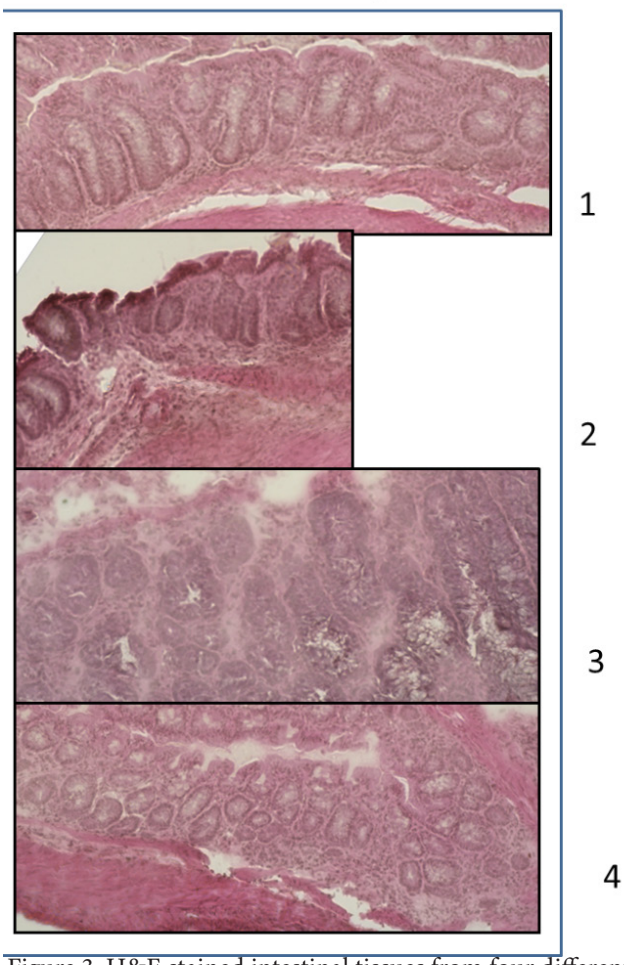
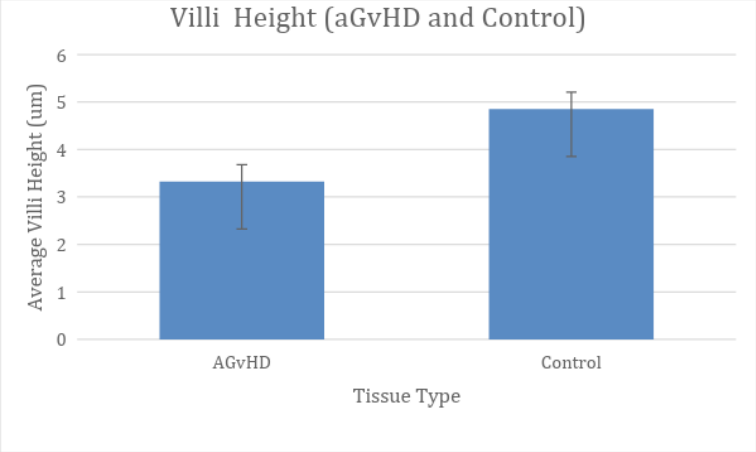


Figure 3. H&E stained intestinal tissues from four different aGvHD mice demonstrating abnormal architechtrue

that plasma FS levels are markedly elevated in patients with aGvHD, especially those with an increased risk of death. However, the tissue source of circulating FS in aGvHD is not known. The next step in this evolution will be to discover the biological mechanism of FS increase and the origin of FS in blood. This study is initial work that lays the foundation for future mechanistic studies of the role of FS in aGvHD.

In this study, we found stark differences in the tissue histology of our samples. Specifically, villi appear to be shorter in length and much more unorganized in aGvHD than in control samples. The lack of organization was statistically confirmed as inter-villi distance in aGvHD samples varied much more than that of control samples ($p<0.01$, F-test, Rstudio). This indicates that there may be an abnormality in organizational proteins that give rise to villi. Now that our model is established and shows consistent differences in histology, tissues can be stained for FS, and FS signaling pathways can be interrogated using molecular arrays. This work is planned for next semester.

The finding of disorganized crypts in aGvHD mice is of particular interest to us, and we have begun to explore this concept of tissue organization further. In recent unpublished data from our lab, the ephrin receptor signaling pathway is greatly enriched in aGvHD tissue in comparison to control samples. Specifically, we found that five proteins (Src, Fak, RhoA, PAK1, and Beta actin) belonging to the pathway have a 97.62 fold enrichment in aGvHD (Holtan, unpublished data). This is important as ephrin plays a large role in the organization/architecture of intestinal tissues. To elaborate, Eph receptors and ephrin ligands signaling helps determine cell positioning along the crypt axis of the small intestine. Eph receptors remodel actin



Average Villi Length (μm)

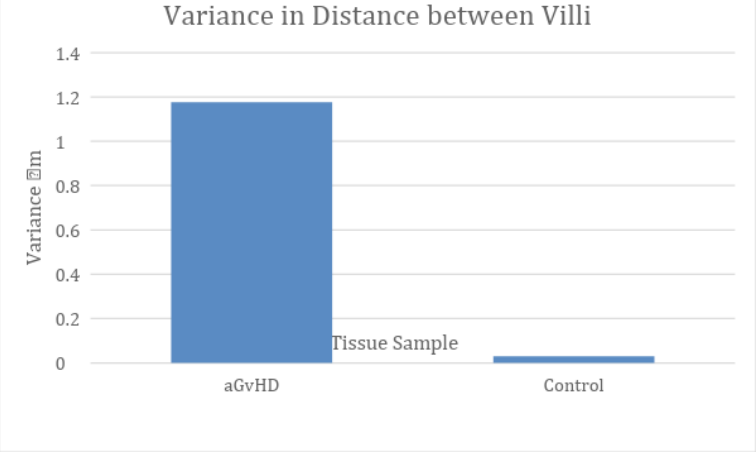
	Mean	Standard Error
aGvHD	3.33	0.35
Control	4.85	1.69

Figure 4. Data on the villi height differences for aGvHD(n=4) versus control tissues (n=4)

cytoskeletons in order to control cell shape and migration in this area. Eph/ephrin signaling also regulates cell to matrix adhesion. That is, ephrin can inhibit FAK, which leads to weak focal adhesions of cells to the intestinal cell matrix (Battle et al., 2003). So, the altered expression of ephrin in aGvHD could lead to changes in cell adhesion and positions along the small intestine. It is possible that the ephrin signaling pathway could have resulted in inter-villi variance that we have observed. Thus, this data gives reason to further study the relationship of ephrin to aGvHD. Furthermore, interactions between FS and ephrin are not known. Given the role of FS in tissue regeneration, it is possible that FS and ephrin may work together to maintain tissue homeostasis.

Although aGvHD villi appeared to be shorter in size (3.32 μm) than control samples (4.85 μm), we did not find this difference to be statistically significant (p=0.384, Two-Sample Heteroscedastic T-test, Rstudio). However, this lack of significance may be due to the extremely small sample size; we could only measure average villi length of 3 aGvHD and 3 control samples as aGvHD sample 4 and control Sample 2 did not have transverse villi sections to measure in length. Prior research would suggest that villi should be statistically shorter in length as growth factors, such as FS, may have differential expression in patients with aGvHD. In the future, we would need a larger sample size to determine whether the difference in length may actually be significant and to determine the tissue staining of FS in aGVHD versus control tissues. With our model now well established, we can begin this work.

The abnormal architectural structure of intestinal villi in aGvHD is very problematic as the capillaries present in this area are responsible for a large majority of nutrient absorption. In fact, jejunal villi can absorb nutrients at rates several hundred times greater than capillaries in the brain and skeletal muscle. The quintessential components of villi for these absorption processes are (1) the permeability surface area of the villus and (2) dynamic increase of villus capillary blood flow. First, nutrients are absorbed by the epithelium and subsequently pass through the capillaries in the lamina propia of the villi. The long shape and large surface area in healthy human villi allows for the rapid absorption of nutrients. Thus, when the normal structure of villi is disrupted by aGvHD, we would expect to see signs and symptoms of



Variance in Distance Between Villi (μm)	
aGvHD	1.18
Control	0.03

Figure 5. Data on the variance in distance between the villi for aGvHD murine tissue samples (n=4) and control samples (n=4)

malabsorption such as decreased nutrient absorption and diarrhea (Pappenheimer et al., 2003).

This is in fact what we see in aGvHD. That is, gastrointestinal aGVHD is defined by the large amount of secretory diarrhea, and for many patients, intestinal damage can lead to a disrupted intestinal barrier and increased risk for infections beyond that caused by immunosuppression alone. Patients with who develop such bacterial translocations consequently have significantly higher mortality rates than those without any infection (Hou et al., 2013). Thus, our finding that aGvHD tissue differs histologically from control samples is further enforced by clinical evidence from previous studies and suggests that lack of intestinal repair may contribute to poor outcomes.

As we have determined that aGvHD impacts villi structure in such a way that patient's health may be affected, further study into factors that account for the disorganization of villi in aGvHD is necessary. That is, it would be beneficial to study the mechanisms behind and increased plasma FS levels (observed in humans, with studies ongoing in mice) and increased ephrin signaling (enriched in mouse array data).

A major limitation of this study was the one semester time constraint, as this investigation consists of multiple parts that could not be all completed. However, we were able to complete Part I: the H&E stains through which we have a foundation to study the effects of FS on aGvHD. The next logical progression to elucidate the mechanism behind increased FS plasma levels in aGvHD would be to run (1) an immunohistochemistry on FS in aGvHD tissue versus normal tissue to determine the localization of FS and (2) to determine whether there is a correlation between the amount of FS present in the tissue and plasma in aGVHD versus controls. Although aGvHD of the intestinal tract is the major cause of mortality in patients with aGvHD, we recognize that the damaged intestine may not be the sole source of circulating FS in aGvHD. Thus, it may become necessary to stain multiple tissue types to determine FS expression (e.g., liver, skin, lung). Finally, we plan to (3) study whether any connection between FS and ephrin signaling exists as it relates to intestinal restitution after severe injury. Such a finding would indeed be novel and possibly suggest new avenues for treatment or supportive care for this devastating disease.

In conclusion, our study demonstrates that there are both qualitative and quantitative changes in the intestine after aGvHD. Additional studies to confirm these findings followed by immunohistochemistry, protein expression arrays, and functional studies of inflammatory neoangiogenesis and tissue regeneration (i.e., studies that focus on host tissue repair as opposed to a solitary focus on donor T cells) are warranted.

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