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14. ABSTRACT
This project is designed to dissect out the primary event that initiates the alteration of epithelial cell homeostasis in inflammatory bowel disease (IBD). Our hypothesis is that activation-induced cytidine deaminase (AID, a DNA-modifying enzyme), which is ectopically expressed in epithelial cells only under intestinal inflammatory condition, is primarily responsible for the initiation of epithelial homeostatic alteration through epigenetic modification. Throughout this project, we have successfully developed fate-mapping double reporter mouse system that allows us to closely examine epithelial cells with prior AID expression versus those without it. By utilizing this mouse system, we have found that AID is expressed by some epithelial crypts under acute intestinal inflammatory condition induced by administration of dextran sulfate sodium (DSS), whereas it is expressed by majority of epithelial crypts during a chronic phase of inflammation. We have also found a possible involvement of AID in the epigenetic modification of some specific genes such as signaling transducers and activators of transcription 3 (STAT3). We initially hypothesized the deleterious role of AID in colitis, but our new data rather suggest the protective role. These findings not only suggest an unexpected function of AID but also have a potential to provide a rationale for the development of novel therapeutic strategy for saving the lives of patients with IBD.

15. SUBJECT TERMS
activation-induced cytidine deaminase, epithelial cell homeostasis, Inflammatory bowel disease

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INTRODUCTION:

Inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) is a chronic intestinal disorder that is caused by dysregulated host/microbial interactions (1,2). Intestinal epithelial cells (IECs), which provide the first line of defense against enteric microorganisms, are responsible for maintaining the appropriate host/microbial interactions (3). Despite the rapid turnover, an intact layer with a constant number of epithelial cells is maintained under healthy steady state. In contrast, the epithelial homeostasis is disrupted in IBD (4). Although many cytokines and growth factors have been demonstrated to participate in the alteration of epithelial homeostasis under intestinal inflammatory conditions, the primary event involved in the "initiation" of this alteration still remains unknown. In this project, we hypothesize that a DNA enzyme, activation-induced cytidine deaminase (AID), which can be ectopically expressed by epithelial cells under inflammatory conditions, is responsible for the initiation of epithelial homeostatic alteration through modification of chromatin status to enhance the accessibility of major transcriptional factors to their promoter regions. The AID-dependent alteration of epithelial homeostasis may contribute to the exacerbation of IBD. This grant proposal is designed to develop robust preliminary data that can be used as a foundation for future research projects to fully prove our hypothesis.

BODY:

Task 1: To investigate whether AID modifies the DNA methylation profiling and the chromatin accessibility in epithelial cells under intestinal inflammatory condition (months 1-18).

1a was designed to expand mouse colony of KI/+ and KI/KI mice:

This 1a was designed to generate a double fluorescent Cre reporter knockin mouse system. In this system, cells express red fluorescent protein, and the color can be permanently changed to green once activation-induced cytidine deaminase (AID) is expressed (5,6). Therefore, this system allows us to distinguish epithelial cells with prior history of AID expressions from those without prior AID expression. As indicated in our progress report, we have successfully established and expanded these mouse strains during the first budget year.

1b. was designed to correct DNA from epithelial cells from KI/+ mice (20 mice) and KI/KI mice (20 mice):

By employing a 30mM EDTA perfusion method that allows us to isolate colonic epithelial cells as an intact crypt unit (7), we have successfully established a technique to purify red fluorescent $(RFP)^+$ epithelial crypts and green fluorescent $(GFP)^+$ epithelial crypts separately from same individual colon after exposure to dextran sulfate sodium (DSS) that induces acute colitis. During the first budget year, we have successfully collected DNA from three types of epithelial cells in the DSS-induced acute colitis model, including RFP^+ KI/+ epithelial cells that have no prior expression of AID, GFP^+ KI/+ epithelial cells that have prior expression of AID, GFP^+ KI/KI epithelial cells that express non-functional AID.

1c was designed to perform methylation-specific PCR (MSP) and EpiTect Methyl qPCR arrays and evaluate the data for future grant (months 7-18):

We performed the methylation-specific PCR to see whether DNA methylation profiling is altered depending on the expression of AID. Unexpectedly, we found in DSS-induced acute colitis model that the majority of inflammation-associated molecules had already been demethylated in epithelial cells before AID expression. Indeed, there were only few molecules whose promoter regions were demethylated depending on the AID expression. One molecule was STAT3 (signaling transducers and activators of transcription 3) that is required for the homeostasis of epithelial cells (see our progress report). Another molecule was chitinase 3 like 1 (CHI3L1) (Figure.1) that is required for the interaction of epithelial cells and potentially pathogenic enteric bacteria (8).

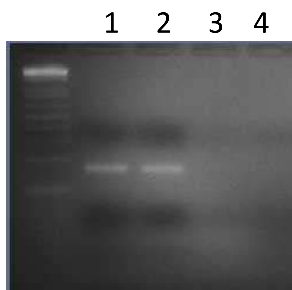

Figure 1:

Methylation-specific PCR of CHI3L1 gene was performed using bisulfite-treated DNA from CpG methylated NIH 3T3 mouse genomic DNA (3T3, as control, lanes 1 and 3) and from RFP(+) colonic epithelial cells (lanes 2 and 4) of reporter mouse. Methylated CHI3L1 bands are shown in lanes 1 and 2 and demethylated CHI3L1 bands are shown in lanes 3 and 4.

Task 2: To investigate the role of ectopic AID expression by epithelial cells in colitis (months 1-18)

2a was designed to develop and expand the mouse colony of RAG1-deficient KI/KI mice (months 1-6).

In order to test the role of AID in the pathogenesis of colitis, we proposed to use a CD45RB model in which colitis is induced in recombinant activation gene (RAG)1 knockout mice versus AID-deficient (KI/KI) RAG double knockout mice by adoptive transfer of purified $CD4^+$ $CD45RB^{high}$ T cells from the spleen of WT mice. As reported in our progress report, we generated the AID-deficient (KI/KI) RAG double knockout mice during the first budget period.

2b and c were designed to purify $CD4^+$ $CD45RB^{high}$ T cells from the spleen of WT mice (60 mice) and transfer them into RAG1-deficient KI/KI mice (15 mice) versus RAG1-deficient KI/+ mice (15 mice) and analyze the development of colitis in the recipient mice (months 9-15)

During second budget year (5 months), we transferred $CD4^+$ $CD45RB^{high}$ T cells from the spleen of WT mice into RAG1 knockout mice (4 mice) versus the newly generated AID-deficient RAG1 double knockout mice (*RAG1-deficient KI/KI mice*, 4 mice) to test whether absence of AID in epithelial cells alters the development of chronic colitis. Unexpectedly, 2 out of 4 AID-deficient RAG1 double knockout recipients died before the scheduled date (8 weeks after cell transfer), whereas all RAG1 knockout recipients survived. In addition, the survived AID-deficient RAG1 double knockout recipients developed more severe colitis as compared to RAG1 knockout recipients. As shown in Figure 2, more severe inflammatory cell infiltration with epithelial cell hyperplasia was seen in the colon of AID-deficient RAG1 double knockout mice with reconstitution of $CD4^+$ $CD45RB^{high}$ T cells (right panel). In contrast to our initial hypothesis (pathogenic role of AID in colitis), these findings suggest that the expression of AID in epithelial cells rather plays a protective role in this chronic colitis. In order to confirm this unexpected finding, we attempted additional cell transfer experiments. However, due to a breeding problem with AID-deficient RAG1 double knockout mice, we were unable to conduct the additional experiments during the rest of second budget year, thus hampering us to statistically evaluate this data.

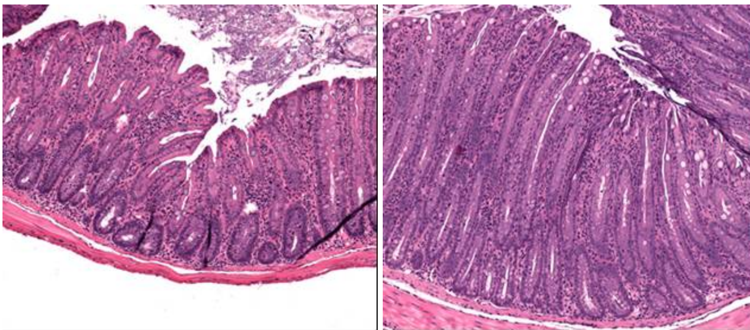

Figure 2:
Histology of colon in RAG1 knockout mouse (left panel) versus AID-deficient RAG1 double knockout mouse (right panel) after transfer of splenic $CD4^+$ $CD45RB^{high}$ T cells from WT mice is shown. More sever inflammation is seen in right panel.

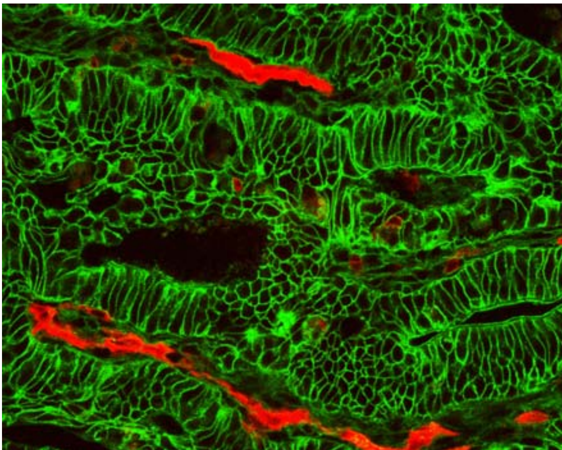

Figure 3:
Fuluorescent image of colon from recipient RAG1-deficient KI/+ double reporter mouse in which cells with history of AID expression express green-fluorescent protein (GFP) and cells without history of AID express red-fluorescent protein. All epithelial cells expressed AID as indicated by expression of GFP.

We next induced colitis in recombinant activating gene (RAG) $1^{-/-}$ mice crossed with double fluorescent Cre reporter knockin mice (RAG1-deficient KI/+ mice) by adoptive transfer of splenic $CD4^+$ $CD45RB^{high}$ T cells from WT mice. This double fluorescent Cre reporter knockin mice allowed us to see AID-expressing epithelial cells (as indicated by GFP expression). As shown in our grant proposal, DSS-induced acute colitis model exhibited both AID(-) (as indicated by RFP expression) and AID(+) (as indicated

by GFP expression) epithelial cells. In contrast, GFP (AID) was expressed by almost majority of epithelial cells in the chronic colitis developing in the CD45RB model (Figure 3). This result has raised two possibilities. First possibility is that AID expression pattern is different between acute and chronic colitis. Alternatively, it is it is equally possible that AID expression pattern differs depending on the time course of colitis, because the AID expression was examined at one time point (8 weeks after adoptive transfer of $CD4^{+}$ $CD45RB^{high}$ T cells).

Since the preliminary data obtained from this project is very attractive both experimentally and conceptually, it should be very important to further extend this project for providing a rationale to develop novel therapeutic strategy to improve the lives of patients with IBD. Since I have recently moved from Massachusetts General Hospital, Boston, USA to Kurume University School of Medicine, Kurume, Japan, I am currently applying some grants in Japan to further extend and fully complete this project. In addition, a collaborator in USA is also seeking a grant support to test the role of AID-dependent expression of chitinase 3 like 1 in inflammatory bowel disease.

KEY RESEARCH ACCOMPLISHMENTS:

Establishment of mouse colony for double reporter mouse system that allows us to purify epithelial cells with a prior history of AID expression versus those without it from a same individual mouse

Establishment of a technique necessary to purify these two epithelial cell groups as a crypt unit

Identification of STAT3 and chitinase 3 like 1 as potential targets of AID

Establishment of AID-deficient RAG double knockout mouse colony to dissect out the role of AID in the pathogenesis of inflammatory bowel disease (IBD)

Identification of potential protective role of AID expressed by epithelial cells in a chronic colitis model (CD45RB model)

Identification of the different expression pattern of AID in acute versus chronic colitis

REPORTABLE OUTCOMES:

Mouse strains: fate-mapping double reporter mouse strains with or without RAG expression and AID-deficient RAG1 double knockout mouse strain

CONCLUSION:

This project was designed to test whether a DNA enzyme AID is involved in the pathogenesis of inflammatory bowel disease through controlling the epigenetic modification in colonic epithelial cells. Throughout this project, all tools (including double reporter mice and AID-deficient RAG1 knockout mice) necessary to test our hypothesis have been successfully developed. In addition, we have identified two potential AID-targeting molecules, STAT3 and chitinase 3 like 1. We also found a possibility that the ectopic expression pattern of AID in epithelial cells differs in acute versus chronic colitis. Importantly, we found a potential protective role of the epithelial AID in a chronic colitis developing in CD45RB colitis model. We believe that this study, if fully completed, would have a potential to open a new avenue for developing a novel therapeutic strategies to improve the lives of patients with IBD e.g. by enhancing the activation of epithelial AID.

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APPENDICES: None