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and through its direct effect on malignant cells (40). Interactions between tumor and immune cells regulate tumorigenesis. IL23/IL17 signaling has been correlated with promotion of tumor growth, as well as IBD pathogenesis. In the tumor microenvironment, IL23/IL17 signaling suppresses antitumorigenic immune response during tumor initiation, growth, and metastases (6–8). In the present study, Cirp deficiency decreased the production of TNFα and IL23 in inflammatory cells and attenuated DSS-induced colitis. In the murine CAC model, we found that TNFα, IL23, and IL17 expression were increased in Cirp-deficient mice and that Cirp was required for inflammation-associated colonic carcinogenesis. Cirp expression was positively associated with the levels of TNFα and IL23 in the colonic mucosa of patients with IBD. Increased Cirp expression, seen in refractory IBD, would promote tumorigenesis by enhancing TNFα and IL23 production. Given the contribution of Cirp in hematopoietic cells to tumor formation (Fig. 6), Cirp likely promotes tumorigenesis through its action in inflammatory cells.

Adult somatic stem cells of the colon sustain self-renewal and are targets for cancer initiation (41), and perturbation in stem cell dynamics is generally considered the first step toward colon tumorigenesis. High levels of stemness factor Sox2 expression are associated with poor prognosis and recurrence in patients with colorectal cancer (42). In patients with IBD, mucosal Cirp expression correlated with the expression of Sox2. We also showed that Cirp is important for sustained expression of Sox2 in the colonic mucosa during colorectal carcinogenesis. Cirp deficiency decreased the number of cells positive for an intestinal cancer stem cell marker Dclk1 at the tumor base. These data suggest a possible function of Cirp in influencing stem cell behavior.

Cancer stem cells, the microenvironment, and the immune system interact with each other through cytokines. In the context of chronic inflammation, cytokines, secreted by immune cells, activate the necessary pathways required by cancer stem cells (43). The number of $Sox2^+$ and $Dclk1^+$ cells in tumor was decreased upon Cirp deletion in the hematopoietic compartment (Fig. 6), suggesting that the absence of Cirp in inflammatory cells decreased production/secretion of these cytokines. There were statistically significant relationships between TNFα and Dclk1 expression and between IL23/IL17 and Sox2 expression in colonic mucosa of patients with ulcerative colitis (data not shown). Thus, Cirp-driven immune responses such as activation of TNFα and IL23/IL17 signaling would affect proliferation of stem cells and increase the expression of stem cell markers. It should be noted, however, that the direct causal link between Cirp and the stem cell markers has not been established in this study. In this regard, the reduced expression of the stem cell markers, such as Dclk1 and Sox2, seen in the absence of Cirp might be due to the secondary effects associated with reduced inflammation.

Apoptotic cell death has been implicated as a major homeostatic and pathogenic mechanism of the intestinal epithelium (2). The lower susceptibility to apoptosis observed in the $Cirp^{-/-}$ intestinal epithelial mucosa in our *in vivo* experiment was unexpected because a previous report showed that Cirp attenuates TNFα-mediated apoptosis by activating ERK and NF-κB in murine embryonic fibroblasts (27). However, expression of Cirp did not affect the sensitivity of murine embryonic fibroblasts to busulfan, and the numbers of apoptotic testicular cells was not different between $Cirp^{-/-}$ and WT mice after busulfan treatment (28). Thus, the role of Cirp may vary depending on cell type and kind of stimuli. In fact, in the DSS-treated colon, Cirp deficiency did not attenuate ERK activity (Supplementary Fig. S4A). In $Cirp^{-/-}$ mice, more inflammatory cells died because of decreased Bcl-2 and Bcl-xL expression than in WT mice (Fig. 4G and H), which would attenuate inflammatory response in $Cirp^{-/-}$ mice. Cell death and inflammation are intimately linked through a self-amplifying loop, making it difficult to distinguish between causes and effects. The attenuated mucosal immune activity due to augmented apoptosis of inflammatory cells likely contributed to the decreased apoptosis of epithelial cells in Cirp-deficient colon.

Bcl-2–mediated apoptosis resistance in inflammatory cells has been shown to attenuate therapeutic efficacy and exacerbate inflammation in IBD (33, 34). In chronically inflamed mucosa seen in refractory ulcerative colitis, Cirp expression is induced in inflammatory cells, which likely inhibits the apoptosis of inflammatory cells, augments proinflammatory cytokine production and treatment resistance via the upregulation of Bcl-2 and Bcl-xL expression. Thus, persistent inflammation resulting from insufficient treatment might further drive resistance to therapy through increased expression of Cirp and subsequent attenuated apoptosis in inflammatory cells. Hypoxia that is enhanced in chronic inflammatory diseases, including IBD, upregulates Cirp expression by a mechanism that involves neither hypoxia-inducible factor (HIF)1 nor mitochondria (20). This may be one explanation for Cirp induction by chronic inflammation. However, the exact mechanisms by which long-term inflammation upregulates Cirp expression remain to be elucidated.

It has been reported that Cirp released into the circulation stimulates the release of TNFα from macrophages via TLR4 and NF-κB activation and triggers an inflammatory response to hemorrhagic shock and sepsis (25). Here, we have shown that in bone marrow–derived macrophages, the presence of Cirp increased IκBα phosphorylation. NF-κB activation would be one of the mechanisms by which Cirp produces proinflammatory cytokines such as TNFα, IL17, and IL23 and upregulates expression of antiapoptotic genes such as Bcl-2 and Bcl-xL (Figs. 4 and 5). A recent study reported the involvement of Cirp in regulating expression of IL1β, another NF-κB target gene, in cultured fibroblasts (44). In bone marrow–derived macrophages, *IL1β* mRNA level was decreased in the absence of Cirp (data not shown). Although in DSS-induced colitis, Cirp protein was not detected in the blood (data not shown), it is conceivable that Cirp released from injured epithelial cells could function as damage-associated molecular pattern molecules *in situ* to activate NF-κB in immune cells of the colon. Furthermore, Cirp can bind the 3′untranslated region of specific transcripts to stabilize them and thus facilitate their transport to ribosomes for translation (22–24). Cirp might regulate the expression of cytokines and antiapoptotic genes posttranscriptionally as well.

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Given the long-term impact of the natural history and treatment of IBD, cancer risk is a major lifelong concern for patients and gastroenterologists. Early detection of CAC/dysplasia is typically achieved by colonoscopic surveillance with multiple biopsies and alternatively by chromoendoscopy with targeted biopsies of all suspect areas. It has been reported that in patients with extensive colitis, surveillance should start after colonoscopy screening (8–10 years after disease onset) and be performed every 2 years for 20 years, then once or twice a year for the next 10 years of disease duration (45). However, such surveillance programs have a number of limitations such as low yield, high cost, invasiveness, incomplete patient enrollment, sampling variations, and poor agreement in histopathologic interpretation (46). If we can reliably predict an individual's risk of CAC so that surveillance strategies can be appropriately personalized, surveillance programs would make much progress. A number of molecular markers for predicting CAC have been reported (47–49) but are not feasible yet for the practical management of patients with IBD. Here, we showed significantly increased Cirp expression in mucosal specimens from patients with refractory IBD that is reported to be associated with increased cancer risk (9). Furthermore, in the murine CAC model, longstanding colonic inflammation increased Cirp expression, which led to enhanced AOM/DSS-induced colorectal tumorigenesis. Cirp expression reflects the presence of refractory inflammation and is therefore a potential marker for predicting the risk of CAC development. Analyzing the Cirp level in colonoscopy specimens may increase the identification rate of IBD patients with a high risk for developing CAC. A future large-scale study of Cirp in IBD patients with different duration and anatomical extent of the disease will be crucial for determining whether Cirp status can be used to predict the risk of cancer and prognosis of patients with IBD.

Taken together, Cirp, whose expression is upregulated by chronic inflammation in humans and mice, enhances the inflammatory response and tumorigenesis by increasing Bcl-2 and Bcl-xL expression and TNFα and IL23/IL17 production in inflammatory cells. Suppression and measurement of Cirp expression is a promising approach for advanced treatment and personalized management of patients with IBD.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Sakurai
Development of methodology: T. Sakurai, J. Fujita
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Sakurai, H. Kashida, T. Mizushima, H. Iijima, N. Nishida
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Sakurai, N. Nishida, J. Fujita, M. Kudo
Writing, review, and/or revision of the manuscript: T. Sakurai, H. Kashida, T. Watanabe, J. Fujita, M. Kudo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Sakurai, S. Hagiwara
Study supervision: T. Sakurai, H. Kashida, H. Higashitsuji, M. Kudo

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Regulation of anergy-related ubiquitin E3 ligase, GRAIL, in murine models of colitis and patients with Crohn's disease

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Abstract

Background Abrogating tolerance is a critical step in the pathogenesis of Crohn's disease (CD). T cell-anergy is one of the main mechanisms of tolerance and is regulated by the gene related to anergy in lymphocytes (GRAIL). This study investigated the expressions and regulation of GRAIL in CD and murine colitis models.

Methods Expressions of GRAIL mRNA and protein in $CD4^+$ T cells were investigated in the peripheral blood and mucosal tissues of patients with CD, mice with dextran sodium salt (DSS)-induced colitis, and *Il-10*-deficient mice. MicroRNAs responsible for the regulation of GRAIL were examined by miRNA microarray. GRAIL-overexpressing T cells were intravenously injected in mice with DSS-induced colitis.

Results The GRAIL expression was higher in the lamina propria (LP) $CD4^+$ T cells of CD patients than of the control subjects, while it was lower in the peripheral blood $CD4^+$ T cells of the CD patients than of the control subjects. The GRAIL mRNA expression was lower, but the GRAIL protein expression was higher in the LP of colitic mice than that of non-colitic mice. The miRNA microarray identified miR-290-5p as an miRNA that inhibits expression of the GRAIL protein and that is highly expressed in the LP of non-colitic mice. GRAIL-expressing T cells expressed regulatory T cell markers and showed suppressive effects in murine DSS-induced colitis.

Conclusions Our results show that expression of GRAIL is uniquely regulated by the specific miRNA in the intestinal mucosa, and suggest that GRAIL may associate with the pathophysiology of CD.

Keywords Inflammatory bowel disease · MicroRNA · GRAIL · Regulatory T cells

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Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are two major categories of inflammatory bowel diseases (IBD) in the human gastrointestinal tract. Both genetic susceptibility and dysregulation of mucosal immune responses against enteric host flora have pivotal roles in mucosal injury [1–3], and abrogating tolerance against unidentified antigens is a critical step in the pathogenesis

of IBD [4]. Clonal anergy of T cells is one of the main mechanisms of tolerance [5], and failure to induce clonal anergy is considered to be associated with the pathogenesis of IBD [6, 7]. T-cell anergy is tightly regulated by E3 ubiquitin ligases, including the gene related to anergy in lymphocyte (GRAIL), the casitas B lineage lymphoma (cbl) family, itchy homologue E3 ubiquitin protein ligase (Itch), and the neural precursor cell expressed developmentally downregulated 4 (NEDD4) [8]. Among these, the role of GRAIL in T-cell anergy has been most vigorously investigated. GRAIL is a type I transmembrane protein that localizes to the endocytic pathway and bears homology to the 'really interesting new gene' (RING) zinc-finger proteins [8]. GRAIL has been shown to be expressed in anergic $CD4^+$ T cells [5, 8]. The expression of GRAIL in $CD4^+$ T cells induces T-cell anergy by limiting interleukin (IL)-2 and IL-4 production. When DO11.10 T cells were infected with an ecotropic retrovirus constitutively expressing wild-type GRAIL, their proliferative capacity in response to antigen and antigen-presenting cells was diminished. Recent studies revealed that GRAIL inhibits the T cell activation cascade by the ubiquitination of CD40 ligand (CD40L), CD83, and T cell receptor (TCR)-CD3 [9–11]. In addition to the importance of GRAIL in converting T cells to an anergic phenotype, a recent study showed that the forced expression of GRAIL in DO11.10 T cells was sufficient for the conversion of these cells to a regulatory phenotype [12]. Additionally, in a Staphylococcal enterotoxin B (SEB)-mediated model of T cell unresponsiveness in vivo, the SEB-exposed forkhead box P3 $(FoxP3)^+GRAIL^+$ T cells were shown to be highly suppressive and non-proliferative, independent of CD25 expression level and glucocorticoid-induced, tumor necrosis factor receptor-related protein (GITR) [13, 14]. This model system revealed a novel paradigm for chronic, non-canonical TCR engagement leading to the development of highly suppressive $FoxP3^+GRAIL^+CD4^+$ T cells. In fact, GRAIL-deficient ($grail^{-/-}$) mice exhibit a susceptibility to autoimmune disease [11]. The importance of GRAIL in human diseases was further demonstrated by our recent study showing that GRAIL expression was increased in the peripheral blood $CD4^+$ T cells of UC patients in remission compared to those of healthy subjects and active UC patients [15]. In addition, GRAIL expression was increased after the effective treatments for active UC patients. To date, no previous reports have investigated the expression of GRAIL in patients with CD and animal models of IBD. In addition, GRAIL expression has not been investigated in the intestine, which comprises the largest pool of immune cells in the human body [16]. We demonstrate here for the first time the expression of GRAIL in patients with CD and in murine colitis models. We also present a novel mechanism in terms of the regulation of GRAIL in the intestine by a specific microRNA (miRNA).

Materials and methods

Human samples

Blood samples were obtained from CD patients who were hospitalized in Osaka University Hospital or from healthy volunteers (HV) recruited in Osaka University Hospital. Intestinal tissues were obtained from the patients with CD or patients with colon cancer who were subjected to surgical resection of the small intestine or the colon in Osaka University Hospital. Patients were diagnosed as having CD according to the endoscopic, radiologic, histological, and clinical criteria provided by the International Organization for the Study of Inflammatory Bowel Disease [17, 18]. The disease activity of CD was evaluated by the Crohn's disease activity index (CDAI) [19]. A CDAI above 150 was defined as active, whereas a CDAI $\leq$150 was defined as remission.

Mice

C57BL/6J mice were purchased from SLC (Shizuoka, Japan). *Il-10*$^{-/-}$ mice (C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To generate dextran sodium sulfate (DSS) colitis, we orally administered 2 % DSS to female C57BL/6J mice (age 8–12 weeks) in their drinking water for 7 days. All mice were kept under specific pathogen-free conditions in an environmentally-controlled, clean room at the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine. The spleen (SP) and mesenteric lymph node (MLN) were aseptically extracted, and single-cell suspensions were prepared by a standard mechanical disruption procedure [20, 21]. Mononuclear cells in the intestinal lamina propria (LP) were prepared by an enzymatic dissociation, as described previously [20, 21]. Briefly, after Peyer's patches were removed from the intestine, the epithelial cell layers were removed from intestinal tissue by incubation in RPMI1640 (Sigma Aldrich, St. Louis, MO, USA) containing ethylenediamine tetraacetic acid (EDTA) [20]. The specimens were dissociated in RPMI1640 containing collagenase (Wako, Osaka, Japan) by stirring at 37 °C. Mononuclear cells were isolated using the discontinuous density gradients procedure with Percoll (GE Healthcare, Pewaukee, WI, USA).