Stress Response Protein Cirp Links Inflammation and Tumorigenesis in Colitis-Associated Cancer

Toshiharu Sakurai¹, Hiroshi Kashida¹, Tomohiro Watanabe², Satoru Hagiwara¹, Tsunekazu Mizushima³, Hideki Iijima³, Naoshi Nishida⁴, Hiroaki Higashitsuji⁵, Jun Fujita⁵, and Masatoshi Kudo¹

Abstract

Colitis-associated cancer (CAC) is caused by chronic intestinal inflammation and is reported to be associated with refractory inflammatory bowel disease (IBD). Defective apoptosis of inflammatory cell populations seems to be a relevant pathogenetic mechanism in refractory IBD. We assessed the involvement of stress response protein cold-inducible RNA-binding protein (Cirp) in the development of intestinal inflammation and CAC. In the colonic mucosa of patients with ulcerative colitis, expression of Cirp correlated significantly with the expression of TNFα, IL23/IL17, antiapoptotic proteins Bcl-2 and Bcl-xL, and stem cell markers such as Sox2, Bmi1, and Lgr5. The expression of Cirp and Sox2 was enhanced in the colonic mucosae of refractory ulcerative colitis, suggesting that Cirp expression might be related to increased cancer risk. In human CAC specimens, inflammatory cells expressed Cirp protein. Cirp⁻/⁻ mice given dextran sodium sulfate exhibited decreased susceptibility to colonic inflammation through decreased expression of TNFα, IL23, Bcl-2, and Bcl-xL in colonic lamina propria cells compared with similarly treated wild-type (WT) mice. In the murine CAC model, Cirp deficiency decreased the expression of TNFα, IL23/IL17, Bcl-2, and Sox2 and the number of Dclk1⁺ cells, leading to attenuated tumorigenic potential. Transplantation of Cirp⁻/⁻ bone marrow into WT mice reduced tumorigenesis, indicating the importance of Cirp in hematopoietic cells. Cirp promotes the development of intestinal inflammation and colorectal tumors through regulating apoptosis and production of TNFα and IL23 in inflammatory cells. Cancer Res. 74(21): 6119-28, ©2014 AACR.

Introduction

The inflammatory bowel diseases (IBD)—ulcerative colitis and Crohn disease—are thought to result from aberrant activation of the intestinal mucosal immune system (1). Although the pathogenesis of IBD remains unclear, a number of studies have suggested the involvement of abnormal apoptosis in intestinal epithelial cells, resulting from increased production of cytokines, such as TNF, ILs, and IFNs (2). TNFα is a key mediator of inflammation in IBD and has been the primary target of biologic therapies (3). This cytokine induces inflammation by promoting the production of IL1β and IL6, expression of adhesion molecules, proliferation of fibroblasts, activation of procoagulant factors, and cytotoxicity of the acute phase response (4). The IL23/Tp17 (T-helper IL17-producing cell) pathway has been identified to play a critical role in IBD. IL23 has been shown to promote the expansion of a distinct lineage of Tp17 cells that are characterized by production of a number of specific cytokines not produced by Tp1 or Tp2 cells, including IL17A, IL17F, IL21, and IL22 (5). IL23/IL17 signaling enhances the immunosuppressive activity of regulatory T cells and reduces CD8⁺ cells in tumor, leading to enhanced tumor initiation and promotion (6, 7). Recently, a study has suggested that colorectal cancer tissue–derived Foxp3⁺ IL17⁺ cells have the capacity to induce cancer-initiating cells in vitro (8). The most conspicuous link between inflammation and colon cancer is seen in patients with IBD (9), and development of colorectal cancer is one of the most serious complications of IBD, which is also referred to as colitis-associated cancer (CAC; ref. 10). Thus, it is of great importance to improve our understanding of the molecular link between chronic inflammation and CAC to identify a target molecule with therapeutic potential for the treatment of IBD and prevention of CAC.

It is widely accepted that most tumors harbor cancer stem cells, which are crucial for a tumor’s evolutionary capability. Cancer stem cells resemble normal stem cells in their capacity to self-renew and continuously replenish tumor progeny (11, 12). The G-protein–coupled receptor Lgr5 and the polycomb group protein Bmi1 are 2 recently described molecular markers of the self-renewing multipotent adult stem cell populations residing in intestinal crypts that mediate regeneration of the intestinal epithelium (13, 14). Pluripotency-

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Toshiharu Sakurai, Department of Gastroenterology and Hepatology, Kinki University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan. Phone: 81-72-2660221, ext. 3525; Fax: 81-72-3672880; E-mail: sakurai@med.kindai.ac.jp

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Molecular and Cellular Pathobiology

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Abstract

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Introduction

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It is widely accepted that most tumors harbor cancer stem cells, which are crucial for a tumor’s evolutionary capability. Cancer stem cells resemble normal stem cells in their capacity to self-renew and continuously replenish tumor progeny (11, 12). The G-protein–coupled receptor Lgr5 and the polycomb group protein Bmi1 are 2 recently described molecular markers of the self-renewing multipotent adult stem cell populations residing in intestinal crypts that mediate regeneration of the intestinal epithelium (13, 14). Pluripotency-

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associated transcription factors like Sox2 are known to regulate cellular identity in embryonic stem cells. Sox2 expression specifically increased the numbers of stem cells and repressed Cdx2, a master regulator of endodermal identity. In vivo studies demonstrated that Sox2, another member of the SoxB gene family, was a specific, immediate, and cell-autonomous target of Sox2 in intestinal stem cells (15). Sox2 participates in the reprogramming of adult somatic cells to a pluripotent stem cell state and is implicated in tumorigenesis in various organs (16).

Cold-inducible RNA-binding protein (Cirp, also called Cirbp or hnRNP A18) was originally identified in the testis as the first mammalian cold shock protein (17) and is suggested to mediate the preservation of neural stem cells (18). Cirp is induced by cellular stresses such as UV irradiation and hypoxia (19–21). In response to the stress, Cirp, which migrates from the nucleus to the cytoplasm, affects posttranscription expression of its target mRNAs (22–24) and functions as a damage-associated molecular pattern molecule that promotes inflammatory responses when present extracellularly (25). Cirp also affects cell growth and cell death induced by TNFα or genotoxic stress (26, 27). However, the involvement of Cirp in colitis and CAC is not well understood.

Here, we examined whether Cirp plays a role in inflammatory immune responses and tumorigenesis in the gut by using a murine CAC model of Cirp-deficient (Cirp<sup>−/−</sup>) mice and found that Cirp promoted colitis and colorectal tumorigenesis by inhibiting apoptosis and increasing TNFα and IL23 production in inflammatory cells. In patients with ulcerative colitis, refractory inflammation is associated with increased Cirp expression (30). Bone marrow transplantation (BMT) experiments were performed as previously described, with slight modifications (31). Bone marrow from the tibia and femur was washed twice in Hank balanced salt solution, and 10<sup>7</sup> bone marrow cells were injected into the tail vein of lethally irradiated (11 Gy) recipient mice. Eight weeks posttransplantation, the mice were subjected to the murine CAC protocol. Bone marrow cells were grown in culture dishes in the presence of macrophage colony-stimulating factor (M-CSF; 10 ng/mL) and then differentiated to bone marrow–derived macrophages in 10 days. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals. The animal study protocol was approved by the Medical Ethics Committee of Kinki University School of Medicine (Osaka-Sayama, Japan).

Materials and Methods

**Human tissue samples**

In total, 236 colonic mucosa specimens were obtained by endoscopy or surgery from patients with ulcerative colitis, including 67 cases of refractory ulcerative colitis, 98 cases of nonrefractory active ulcerative colitis, and 20 cases in remission, as well as 21 colonic mucosa of patients with Crohn disease and 30 normal colonic mucosa specimens from controls without IBD. Refractory ulcerative colitis was defined according to endoscopic criteria and categorized as being active for more than 6 months. Active inflammation was defined as Mayo endoscopic score ≥2. CAC specimens were obtained from 10 patients who had undergone colorectal resection. The clinical study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the relevant institutional review boards.

**Mice and treatment**

Cirp<sup>−/−</sup> mice showing neither gross abnormality nor colonic inflammation were used as a murine CAC model. The generation of Cirp<sup>−/−</sup> mice has been described previously (28). Sex- and age-matched C57BL/6 wild-type (WT) and Cirp<sup>−/−</sup> mice (8–12 weeks old) received 2.5% (w/v) dextran sodium sulfate (DSS; molecular weight, 36,000–50,000 kDa; MP Biomedicals) in drinking water. Mice were intraperitoneally injected with 20 mg/kg anti-TNFα antibody (#16-7423, eBioscience) or an IgG isotype control before DSS treatment.

Isolation of lamina propria cells was performed as described previously (29). The isolated cells were sorted using immunomagnetic beads coated with monoclonal antibodies against CD11b (MACS Beads, Miltenyi Biotec) with the help of a separation column and a magnetic separator from the same company in accordance with the manufacturer’s recommendations for isolating murine macrophages.

As the protocol for the murine CAC model, mice were intraperitoneally injected with 12.5 mg/kg azoxymethane (AOM; Sigma-Aldrich). After 5 days, 2.0% DSS was included in the drinking water for 5 days, followed by 16 days of regular water. This cycle was repeated 3 times. Then, 1.5% DSS was included in the drinking water for 4 days, followed by 7 days of regular water. Upon sacrifice, the colon was excised from the ileocecal junction to the anus, cut open longitudinally, and prepared for histologic evaluation. Colonos were assessed macroscopically for polyps under a dissecting microscope.

Bone marrow transplantation (BMT) experiments were performed as previously described, with slight modifications (30). Bone marrow from the tibia and femur was washed twice in Hank balanced salt solution, and 10<sup>7</sup> bone marrow cells were injected into the tail vein of lethally irradiated (11 Gy) recipient mice. Eight weeks posttransplantation, the mice were subjected to the murine CAC protocol. Bone marrow cells were grown in culture dishes in the presence of macrophage colony-stimulating factor (M-CSF; 10 ng/mL) and then differentiated to bone marrow–derived macrophages in 10 days. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals. The animal study protocol was approved by the Medical Ethics Committee of Kinki University School of Medicine (Osaka-Sayama, Japan).

**Colonic injury scoring**

Excised colons were rolled up and fixed in 10% formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The degree of colonic injury was assessed by histologic scoring as described previously (31), with minor modifications. The protocol is described in detail in Supplementary Materials and Methods.

**Biochemical and immunohistochemical analyses**

Real-time qPCR, immunoblotting, and immunohistochemistry were previously described (32). Primer sequences are given in Supplementary Materials and Methods. The following antibodies were used: anti-actin and anti-DCAMKLK1 (Dclk1) from Sigma-Aldrich; anti-Bel-2, anti-phospho-IκBα, anti-IκBζ, anti-phospho-ERK, anti-ERK, anti-Sox2, anti-E-cadherin, anti-PCNA from Cell Signaling; and anti-F4/80 from eBioscience. Generation of anti-Cirp polyclonal antibody was previously described (28). Immunohistochemistry was performed using ImmPRESS reagents (Vector Laboratory) according to the manufacturer’s recommendations. Immunofluorescent terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was performed to measure...
apoptosis in paraffin-embedded sections using the In Situ Apoptosis Detection Kit as described by the manufacturer (Takara). Nuclei were stained with 4,6-diamidino-2-phenylindole to count the total cells per crypt. A minimum of 10 crypts were counted per section.

Statistical analysis

Differences were analyzed using the Student t test. To compare variables of more than 2 conditions, ANOVO with post hoc Tukey–Kramer honestly significant difference (HSD) multiple comparison was applied. The relationship between the expression of several genes was analyzed by Spearman rank correlation test. P < 0.05 was considered significant.

Results

**Correlation of Cirp expression with TNFα, IL23/IL17, Bcl-2, and stem cell marker expression in patients with IBD**

Cirp expression correlated weakly but significantly with TNFα with a linear coefficient of 0.26 in the colonic mucosa of patients with ulcerative colitis (Supplementary Fig. S1A). In patients with Crohn disease, Cirp expression did not significantly correlate with TNFα (data not shown) probably because the majority of patients with Crohn disease enrolled in this study had undergone anti-TNF therapy. IL23p19 is the specific subunit of IL23, a positive regulator of Th17 and other IL17-producing cells (5). A significant correlation was found between Cirp and IL23p19 mRNA expression in patients with ulcerative colitis (Supplementary Fig. S1B and S1C) and in patients with Crohn disease (Supplementary Fig. S2A).

Defective apoptosis of inflammatory cell populations regulated by Bcl-2 seems to be a relevant pathogenetic mechanism in IBD (33, 34). There was a significant correlation between Cirp and Bcl-2 expression with a linear coefficient of 0.76 in patients with ulcerative colitis and with a linear coefficient of 0.60 in patients with Crohn disease (Fig. 1A and Supplementary Fig. S2B). Expression of Bcl-xL, another antiapoptotic protein, was significantly correlated with that of Cirp with a linear coefficient of 0.60 in patients with ulcerative colitis (Supplementary Fig. S1D) and with a linear coefficient of 0.85 in patients with Crohn disease (Supplementary Fig. S2C).

Stem cells, characterized by their ability to self-renew indefinitely and produce progeny capable of repopulating tissue-specific lineages, are critical for maintaining normal tissue homeostasis (35). Cirp is suggested to mediate the preservation of neural stem cells (18). Cirp expression correlated with Sox2, Bmi1, Lgr5, and Dclk1 levels with linear coefficients of 0.62, 0.45, 0.42, and 0.25, respectively, in patients with ulcerative colitis (Fig. 1B–D and Supplementary Fig. S1E) and correlated with Sox2 with a linear coefficient of 0.63 in patients with Crohn disease (Supplementary Fig. S2D). Cirp might be involved in regulation of intestinal inflammation and homeostasis maintenance in patients with IBD.

**Increased Cirp expression in the colonic mucosa of patients with refractory ulcerative colitis**

We next explored whether an association exists between Cirp expression and the clinical status of patients with ulcerative colitis. Refractory and nonrefractory active ulcerative colitis could not be distinguished by endoscopic findings (Fig. 2A). Cirp expression levels were specifically increased in patients with refractory ulcerative colitis associated with long-term inflammation, whereas similar expression levels of Cirp were found between normal colonic mucosa and the mucosa of patients with nonrefractory active ulcerative colitis (Fig. 2B). Similarly, increased Sox2 expression levels were found in the colonic mucosa of patients with refractory IBD (Fig. 2C). Immunohistochemistry showed that Sox2 was expressed in the mesenchyme and Dclk1 was expressed in the crypt of patients with refractory ulcerative colitis (Supplementary Fig. S3B). In contrast, increased TNFα expression was found in the colonic mucosa of both refractory and nonrefractory active ulcerative colitis (Fig. 2D). Immunohistochemistry was performed to identify the cells expressing Cirp in the human intestine, and inflammatory cells were found to express more Cirp protein than epithelial cells, whose expression pattern was similar in controls and patients with ulcerative colitis. In chronically inflamed mucosa, Cirp expression was enhanced in inflammatory cells (Fig. 2E and Supplementary Fig. S3B). Inflammatory cells preferentially but not exclusively expressed Cirp protein also in human CAC cases (Supplementary Fig. S3C).

**Cirp−/− mice challenged with DSS have decreased susceptibility to inflammation**

DSS-induced colitis is a murine model resembling human ulcerative colitis. Experimental colitis was induced by treating mice with 2.5% DSS. Histologic analysis revealed substantially less epithelial damage and disruption of crypt architecture in
Cirp expression is increased in the colonic mucosa of patients with refractory ulcerative colitis (UC). A, endoscopic images of refractory and nonrefractory active ulcerative colitis specimens with a magnified inset. B–D, expression of Cirp (B), Sox2 (C), and TNFα (D) mRNA in normal colonic mucosa (control, n = 30), colonic mucosa of patients with nonrefractory active ulcerative colitis (nonrefractory, n = 98), and those with refractory ulcerative colitis (refractory, n = 67), as determined by quantitative real-time qPCR. P values were calculated by post hoc Tukey-Kramer HSD multiple comparison. The F and P values for the ANOVA test are as follows: F(2, 192) = 11.38; P < 0.0001 (B), F(2, 192) = 10.29; P < 0.0001 (C), and F(2, 192) = 13.70; P < 0.0001 (D). E, representative images of immunohistochemical findings in human colonic mucosa of patients without ulcerative colitis and those with nonrefractory and refractory ulcerative colitis. Scale bar, 50 μm.

Cirp−/− mice than in WT mice (Fig. 3A), and inflammatory cell infiltration into the colon was less in Cirp−/− mice (Fig. 3B). Immunohistochemically assessed macrophage infiltration was smaller in Cirp−/− than in WT mice after DSS administration (Fig. 3C), and the epithelial injury score was significantly smaller in the Cirp−/− mice than in the controls (Fig. 3D). Next, we compared apoptosis induction in DSS-treated WT and Cirp−/− mice. Apoptosis detected by TUNEL staining was observed in DSS-treated mice, primarily in the colonic crypts (Fig. 3E), but was blocked by 50% in the Cirp−/− mice (Fig. 3F). Examination of the colonic lysates from DSS-treated WT and Cirp−/− mice showed that the Cirp presence increased PCNA expression in the colon (Supplementary Fig. S4A).

The associated immune response was investigated by analyzing colonic cytokine levels. Colonic tissue from Cirp−/− mice showed a smaller immune response with lower levels of proinflammatory cytokine TNFα and IL23 than that of WT mice (Fig. 4A–C), which is consistent with the data in humans (Fig. 1 and Supplementary Fig. S1). TNFα expression was upregulated in nonrefractory active ulcerative colitis whereas Cirp expression was not in these patients (Fig. 2B and D). This is probably because TNFα is induced in both a Cirp-dependent and -independent manners in the colon. There was no significant difference in IL1β, IL10, and IL21 (Fig. 4B and Supplementary Fig. S4B). To explore the mechanisms of the effects of Cirp on TNFα production, we sought to confirm these findings in vitro. In lamina propria cells isolated from DSS-treated colons of Cirp-deficient mice, expression of TNFα and IL23 was decreased compared with those of WT mice (Fig. 4D). TNFα is produced chiefly by activated macrophages, although it can be produced by many other cell types as lymphocytes and natural killer cells (36), so we next isolated macrophages from DSS-treated colons. TNFα mRNA expression was significantly reduced in Cirp-deficient macrophages (Fig. 4E). Macrophages...
Cirp and Inflammatory Bowel Disease

Figure 4. Cirp contributes to TNF-α and IL23 production and Bcl-2 and Bcl-xL expression in inflammatory cells. A–C, relative levels of TNF-α (A), IL1β (B), and IL23 (C) in colonic tissues from mice treated with DSS, as determined by real-time qPCR (n = 8 per group). Expression level in colonic tissues from nontreated WT mice was set as 1. D and E, effect of Cirp on gene expression in colonic lamina propria (LP) cells (D) and macrophages (E). Lamina propria cells were isolated, and cytokine mRNA expression was analyzed by real-time qPCR. The mRNA expression levels in lamina propria cells from nontreated WT mice were set as 1. F, inflammatory cell infiltration into colonic tissues of WT and Cirp–/– mice 7 days after the initiation of DSS administration with or without anti-TNF-α antibody treatment. Scoring was performed as described in Materials and Methods. n = 3 per group. **P < 0.01 compared with control.

G, effect of Cirp on gene expression in colonic lamina propria cells. Lamina propria cells were isolated, and cytokine mRNA expression was analyzed by real-time qPCR. The mRNA expression levels in lamina propria cells from nontreated WT mice were set as 1. *, P < 0.05 compared with control.

H, representative images of immunohistochemical detection of E-cadherin, a marker for epithelial cells, and TUNEL staining of colonic tissues from DSS-treated mice.

Figure 4A. Relative mRNA levels in colonic tissues from mice treated with DSS, as determined by real-time qPCR. WT and Cirp–/– mice were treated with DSS for 7 days. Expression levels were normalized to the expression levels in nontreated WT mice. Relative levels of TNF-α, IL1β, and IL23 were analyzed. n = 8 per group. P-values were calculated using the Student’s t-test.

Figure 4B. Relative mRNA levels in colonic tissues from mice treated with DSS, as determined by real-time qPCR. WT and Cirp–/– mice were treated with DSS for 7 days. Expression levels were normalized to the expression levels in nontreated WT mice. Relative levels of TNF-α, IL1β, and IL23 were analyzed. n = 8 per group. P-values were calculated using the Student’s t-test.

Figure 4C. Relative mRNA levels in colonic tissues from mice treated with DSS, as determined by real-time qPCR. WT and Cirp–/– mice were treated with DSS for 7 days. Expression levels were normalized to the expression levels in nontreated WT mice. Relative levels of TNF-α, IL1β, and IL23 were analyzed. n = 8 per group. P-values were calculated using the Student’s t-test.

Figure 4D. Relative mRNA levels in colonic tissues from mice treated with DSS, as determined by real-time qPCR. WT and Cirp–/– mice were treated with DSS for 7 days. Expression levels were normalized to the expression levels in nontreated WT mice. Relative levels of TNF-α, IL1β, and IL23 were analyzed. n = 8 per group. P-values were calculated using the Student’s t-test.

Figure 4E. Relative mRNA levels in colonic tissues from mice treated with DSS, as determined by real-time qPCR. WT and Cirp–/– mice were treated with DSS for 7 days. Expression levels were normalized to the expression levels in nontreated WT mice. Relative levels of TNF-α, IL1β, and IL23 were analyzed. n = 8 per group. P-values were calculated using the Student’s t-test.

Figure 4F. Relative mRNA levels in colonic tissues from mice treated with DSS, as determined by real-time qPCR. WT and Cirp–/– mice were treated with DSS for 7 days. Expression levels were normalized to the expression levels in nontreated WT mice. Relative levels of TNF-α, IL1β, and IL23 were analyzed. n = 8 per group. P-values were calculated using the Student’s t-test.

Figure 4G. Relative mRNA levels in colonic tissues from mice treated with DSS, as determined by real-time qPCR. WT and Cirp–/– mice were treated with DSS for 7 days. Expression levels were normalized to the expression levels in nontreated WT mice. Relative levels of TNF-α, IL1β, and IL23 were analyzed. n = 8 per group. P-values were calculated using the Student’s t-test.

Figure 4H. Relative mRNA levels in colonic tissues from mice treated with DSS, as determined by real-time qPCR. WT and Cirp–/– mice were treated with DSS for 7 days. Expression levels were normalized to the expression levels in nontreated WT mice. Relative levels of TNF-α, IL1β, and IL23 were analyzed. n = 8 per group. P-values were calculated using the Student’s t-test.

derived from WT bone marrow exhibited marked upregulation of TNF-α mRNA relative to those derived from Cirp−/− bone marrow (Supplementary Fig. S4C). Cirp has been reported to activate NF-κB (25, 27). Consistently, the presence of Cirp increased 1αβ phosphorylation in bone marrow–derived macrophages (Supplementary Fig. S4D). Treatment with anti-TNF-α antibody reduced inflammatory cell infiltration in WT mice but not in Cirp−/− mice (Fig. 4F). These data indicate that Cirp in inflammatory cells augments the inflammatory response by producing cytokines such as TNFα and IL23. In addition, Bcl-2 and Bcl-xL mRNA expression was significantly reduced in Cirp-deficient inflammatory cells (Fig. 4G), and more apoptosis was found in Cirp−/− immune cells than WT immune cells (Fig. 4H), which might at least partially contribute to the attenuated inflammatory responses by Cirp deficiency.

Cirp deficiency attenuated tumorigenesis in the murine CAC model

Chronic inflammation increases intestinal cancer risk in IBD (10). To investigate the precise pathogenic mechanisms underlying IBD-associated colorectal carcinogenesis, we used the AOM plus DSS mouse model to study the role of Cirp in CAC. In the AOM/DSS protocol, a significant decrease was noted in the number and maximum size of tumors in the Cirp−/− mice compared with WT mice (Fig. 5A and B). Histologic
Cirp deficiency affects colonic tumorigenesis in a murine model of CAC. A, typical examples of macroscopic tumorigenesis in the CAC model. Colon lengths revealed larger adenomas with a complex tumor growth pattern in WT tumors compared with Cirp−/− tumors (Fig. 5C). Extensive infiltration of inflammatory cells into the lamina propria and submucosal layer surrounding the tumors suggest the involvement of inflammatory responses in the tumorigenesis seen in the AOM/DSS-treated mice (Fig. 5C). Colon length was measured as one parameter to assess the severity of inflammation and was found to be significantly longer in Cirp−/− mice than in WT mice (Fig. 5D). The expression of TNFα was decreased in nontumorous tissue, but not in tumors, of Cirp−/− mice challenged with AOM and DSS (Fig. 5E). Tumor and nontumor cells would use different mechanisms to regulate gene expression. In tumor cells, expression of TNFα might be upregulated in a Cirp-independent manner. IL23 and IL17 inhibit antitumor immunity and promote tumorigenesis (6–8). Expression of IL23 and IL17 was decreased in Cirp−/− tumors and nontumor colons compared with WT counterparts (Fig. 5E). Bcl-2 and Bcl-xL mRNA expression that is upregulated by Cirp in inflammatory cells (Fig. 4G) was significantly reduced in Cirp-deficient colons (Fig. 5E). Cirp deficiency decreased proliferating cell nuclear antigen (PCNA) expression in DSS-treated colons, whereas in established tumors, neither apoptosis nor PCNA expression was significantly affected by Cirp deletion (Supplementary Fig. S6). The expression of stemness factor Sox2 was decreased in Cirp-deficient colons.
and tumors compared with WT tissues (Fig. 5E and Supplementary Fig. 5A). Dclk1 is a candidate tumor stem cell marker in the gut (37). Deletion of Cirp decreased the number of Dclk1<sup>+</sup> cells at the tumor base (Fig. 5F). Cirp expression was increased in the colonic mucosa of tumor-harboring mice given DSS and AOM, whereas short-term inflammation induced by DSS administration for 7 days did not upregulate Cirp expression (Fig. 5G and Supplementary Fig. 5B). Coupled with the findings in humans (Fig. 2B and E), these results suggest that Cirp is induced by long-term intestinal inflammation.

Cirp promotes tumorigenesis through hematopoietic cell populations

To functionally characterize the contribution of different cell populations to colorectal tumorigenesis, we created Cirp chimeric mice using a combination of <sup>60</sup>Co-irradiation and BMT. Nontransplanted controls survived less than 2 weeks after irradiation, indicating there was ablation of the endogenous marrow. Transplanted animals were allowed to recover for 2 months before placing them on the AOM/DSS protocol. WT mice rescued with Cirp<sup>–/–</sup> bone marrow had a significantly smaller tumor burden than those rescued with WT bone marrow (Fig. 6A and B). Both WT and Cirp<sup>–/–</sup> mice rescued with WT bone marrow had equivalent tumor sizes (Fig. 6B). Chimeras harboring Cirp<sup>–/–</sup> bone marrow diminished expression of TNF<sub>α</sub> (Fig. 6C), which indicates that Cirp in hematopoietic cells is involved in upregulation of TNF<sub>α</sub>. Cirp-chimeric mice with Cirp-deficient bone marrow showed a smaller number of Dclk1<sup>+</sup> and Sox2<sup>+</sup> cells in tumors than in the WT mice (Fig. 6D and E). Taken together, at least in this model, the absence of Cirp in hematopoietic cellular compartments protects against AOM/DSS-induced tumorigenesis.

Discussion

The association between IBD and colorectal cancer is well established; the cumulative risk of developing colorectal cancer after 20 years is 7% in ulcerative colitis and 8% in Crohn disease (10). Optimal IBD management would reduce the risk of CAC (35). While it is clear that chronic mucosal inflammation plays a causative role in the transition to adenocarcinoma, the molecular link between inflammation and cancer remains to be elucidated. AOM is a procarcinogen that is metabolically activated to a potent alkylating agent that forms O<sup>6</sup>-methylguanine (38). Its oncogenic potential is markedly augmented in the setting of chronic inflammation, such as that induced by repeated cycles of DSS treatment (39). TNF<sub>α</sub>, a key mediator of inflammation in IBD (3), contributes to tumorigenesis by creating a tumor-supportive inflammatory microenvironment.
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 antitumorogenic 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 Dclkl 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 Dclkl 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-xB 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-xB 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 IkBα phosphorylation. NF-xB 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-xB 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-xB 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.
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 colonicoscopy 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, biomechanical, 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. Higashitsujii, M. Kudo

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Toshiharu Sakurai, Hiroshi Kashida, Tomohiro Watanabe, et al.


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