stain collagen$^+$ fibroblasts, we stained tissue sections with anilin blue orange G solution (Muto Pure Chemicals) for 1 hr and counterstained with toluidine blue (Wako) for 20 min. For the staining with anti-mouse cathelicidin antibody (Abcam), tissue sections were retrieved with Retrievagen A (BD Biosciences).

Inflammation severity was scored as follows: 0, no; 1, minimal; 2, mild; 3, moderate; and 4, marked. The slides were blinded, randomized, and reread to determine score. The total score was calculated as the sum of scores for inflammation, neutrophil number, mononuclear cell number, edema, and epithelial hyperplasia (Otsuka et al., 2011).

Whole-Mount FISH

Skin was fixed in 4% paraformaldehyde at 4°C overnight and washed with PBS for 7 hr. Tissues were hybridized with 10 μg/mL of Alexa 488-conjugated DNA probe (EUB338, Invitrogen) in a hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.1% SDS, and 10 μg/mL) at 42°C overnight. After washing twice in a washing buffer (0.45 M NaCl, 20 mM Tris-HCl, 0.01% SDS) at 42°C for 10 min, tissues were flushed with PBS and observed by confocal microscopy (DM IRE2/TCS SP2, Leica) (Obata et al., 2010).

Microarray Analysis

Total RNA was prepared with RNeasy kit (QIAGEN). cRNA was hybridized with DNA probes on a GeneChip Mouse Genome 430 2.0 array (Affymetrix) (Kunisawa et al., 2013). Data were analyzed with GeneSpring 7.3.1 software (Silicon Genetics).

Quantitative RT-PCR

Total RNA was prepared with TRIzol (Invitrogen) and reverse transcribed by Superscript VILO (Invitrogen). Quantitative RT-PCR was performed with the LightCycler 480 II (Roche) and the Universal Probe Library (Roche). Primer sequence is available in Table S1.

Statistical Analysis

Statistical analysis was performed with the unpaired two-tailed Student's t test and Welch's t test. In some experiments, one-way ANOVA and Tukey's method were employed as indicated in figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.01.014.

AUTHOR CONTRIBUTIONS

Y. Kurashima conducted the research, performed experiments, and wrote the manuscript. T.A., K.F., Y. Kogure, Y.S., and E.H. performed gene expression and animal experiments. N.S. conducted in situ experiments. K.K. and A.O. contributed to the experimental design, skin analysis, and histological scoring. M.K., S.A., and S.S. helped construct the transgenic mice. T.S. conducted the morphological analysis of MCs. K.M., J.K., and H.K. supervised the project and wrote the manuscript.

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

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

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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, Bcl-xL, 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/T_H17 (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 T_H17 cells that are characterized by production of a number of specific cytokines not produced by T_H1 or T_H2 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-

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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 Sox21, 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 ($Cirp^{-/-}$) 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 in the colonic mucosa, which would increase the risk for CAC. This study represents the first report of the functional link between Cirp and intestinal tumorigenesis.

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

$Cirp^{-/-}$ mice showing neither gross abnormality nor colonic inflammation were used as a murine CAC model. The generation of $Cirp^{-/-}$ mice has been described previously (28). Sex- and age-matched C57BL/6 wild-type (WT) and $Cirp^{-/-}$ 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 intrapertioneally 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. Colons 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 10^7 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 immunochemical 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-DCAMLK1 (Dclk1) from Sigma-Aldrich; anti-Bcl-2, anti-phospho-IκBα, anti-IκBα, 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