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Positive role of CHOP, a transcription factor involved in the ER stress response in the development of colitis

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Abbreviations used in this paper: ANOVA, analysis of variance; 5-ASA, 5-aminosalicylic acid; ATF, cAMP-dependent transciption factor; BHT, butylated hydroxytoluene; CAM, cell adhesion molecule; CD, Crohn's disease; C/EBP, CCAAT/enhancer binding protein; CHOP, C/EBP homologous protein; DAB, 3,3'-diaminobenzidine; DAI, disease activity index; DAPI, 4',

6-diamidino-2-phenylindole dihydrochloride; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; ERO, endoplasmic reticulum oxidoreductin; ER, endoplasmic reticulum; ESR, Electron Spin Resonance; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP, glucose-regulated protein; H_2DCF, 2',7'-dichlorofluorescein diacetate; H & E, hematoxylin and eosin; HSF, heat shock factor; HTAB, hexadecyl trimethyl ammonium bromide; IBD, inflammatory bowel disease; IL, interleukin; ICAM, intercellular adhesion molecule; IRE, site-specific endoribonuclease; LPS, lipopolysaccharide; MAdCAM, mucosal addressin cell adhesion molecule; MPO, myeloperoxidase; MTT, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NF-κB, nuclear factor kappa B; O.C.T., optimal cutting temperature; ROS, reactive oxygen species; PERK/PEK, protein kinase R-like ER kinase/pancreatic eIF-2alpha kinase; POBN, alpha-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone; S.E.M., standard error means; siRNA, small interfering RNA; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactant substance; TdTase, terminal deoxynucleotidyl transferase; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; TUNEL,

TdT-mediated dUTP-biotin end labelling; UC, ulcerative colitis; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

While recent reports suggest that the endoplasmic reticulum (ER) stress response is induced in association with the development of inflammatory bowel disease (IBD), its role in the pathogenesis of IBD remains unclear. CHOP is a transcription factor involved in the ER stress response, especially ER stress-induced apoptosis. We here found that experimental colitis was ameliorated in CHOP-null mice, suggesting that CHOP exacerbates the development of colitis. The mRNA expression of *Mac-1* (*CD11b*, positive regulator of infiltration of macrophages), *Ero-1α* and *Caspase-11* (positive regulator of production of IL-1β) in the intestine was induced with the development of colitis and this induction was suppressed in CHOP-null mice. ERO-1α is involved in the production of ROS and an increase in the production of ROS associated with development of colitis in the intestine, was suppressed in CHOP-null mice. More apoptotic cells in the intestinal mucosa of wild-type mice than in CHOP-null mice were observed to accompany the development of colitis. These results suggest that up-regulation of CHOP expression exacerbates the development of colitis. Furthermore, this activity of CHOP seems to involve various mechanisms, such as stimulation of macrophage infiltration via the induction of Mac-1, stimulation of ROS-production via

the induction of ERO-1α, stimulation of IL-1β production via the induction of Caspase-11, and stimulation of intestinal mucosal cell apoptosis.

Inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC), have become substantial health problems with an actual prevalence of 200-500 per 100, 000 people in western countries, which almost doubles every 10 years.[1] Although the etiology of IBD is not clear at present, recent studies suggest that IBD is a disorder involving activation of leukocytes (macrophages, lymphocytes and neutrophils) and their infiltration into the inflamed intestine, and intestinal mucosal damage induced by reactive oxygen species (ROS).[2] To understand the molecular mechanism underlying the pathogenesis of IBD and to establish a clinical protocol for its treatment, it is important to identify proteins that are involved in the pathogenesis of IBD. For this purpose, various experimental animal models of colitis, in particular the dextran sulfate sodium (DSS)- and trinitrobenzene sulfonic acid (TNBS)-induced colitis models, are useful.[3]

Pro-inflammatory cytokines and cell adhesion molecules (CAMs) play an important role in the activation and infiltration of leukocytes that are associated with IBD. Increases in the intestinal levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, as well as various CAMs, such as intercellular adhesion molecule-1 (ICAM-1) and Mac-1, have been reported in both IBD patients and

animal models of IBD.[4-11] TNF-α-deficient mice or ICAM-1-deficient mice show a phenotype resistant to experimental colitis.[8,12] A chimeric monoclonal antibody against TNF-α, infliximab, antibody against Mac-1 and alicaforsen (ISIS 2302), an oligodeoxynucleotide that inhibits the expression of ICAM-1, are reported to be effective in the treatment of IBD patients and experimental colitis.[8,10,13-15]

Accumulation of unfolded and misfolded proteins in the endoplasmic reticulum (ER) induces the ER stress response. At the final step of mammalian ER stress response, the apoptotic response is initiated to eliminate cells. C/EBP homologous transcription factor (CHOP) is a transcription factor involved in the ER stress response, especially ER stress-induced apoptosis through various mechanisms such as down-regulation of Bcl-2 and up-regulation of Bim.[16-18] A close relationship between inflammation and the ER stress response, especially the induction of CHOP, has been suggested. For example, TNF-α was reported to induce the ER stress response and expression of CHOP.[19] CREBH was recently identified as a factor connecting the ER stress response and the acute inflammatory response.[20] Therefore, it is reasonable to hypothesize that the ER stress response, and CHOP in particular, is involved in the pathogenesis of IBD. In fact,

some recent reports support this idea; up-regulation of CHOP and GRP78 was observed in the inflamed intestine in both IBD.[21,22] However, the exact role (positive or negative) of the ER stress response (or CHOP) in the pathogenesis of IBD has remained unknown. The analysis of knockout mice is useful in addressing this type of question. For example, we recently suggested, through analysis of DSS-induced colitis in heat shock factor 1 (HSF1, a transcription factor involved in the heat shock response)-null mice, that HSF1 plays a protective role, inhibiting the development of IBD.[23] In the present study, we compared the development of DSS- and TNBS-induced colitis between CHOP-null mice and wild-type mice and obtained genetic evidence that CHOP plays a positive role in the pathogenesis of experimental colitis. Furthermore, results in this study suggest that CHOP achieves this effect through various mechanisms such as stimulation of intestinal ROS production, sensitisation of intestinal mucosal cells to ROS-induced apoptosis, stimulation of macrophage infiltration into the inflamed intestine and stimulation of the intestinal production of IL-1β. Based on these findings, we propose that inhibitors for CHOP may be therapeutically beneficial for the treatment of IBD.

Materials and Methods

Chemicals, Cells and Animals

Paraformaldehyde, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), menadione, fetal bovine serum (FBS), *o*-dianisidine, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF), and TNBS were obtained from Sigma (St. Louis, MO). Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), *n*-butanol and pyridine were from Nacalai Tesque (Kyoto, Japan). DSS (M.W. 5000, 15–20% sulfur content) was from WAKO Pure Chemicals (Tokyo, Japan). Proteose peptone was from Becton Dickinson (San Jose, CA). Lipopolysaccharide (LPS) was from List Biological Laboratories, Inc (Campbell, CA). Antioxidant Assay Kit was from Cayman (Ann Arbor, MI). An enzyme-linked immunosorbent assay (ELISA) kit for the detection of IL-1β was from Pierce Chemical (Rockford, IL). Optimal cutting temperature (O.C.T.) compound was from Sakura Finetek Japan (Tokyo, Japan). Mayer's hematoxylin, 1% eosin alcohol solution and Malinol were from MUTO Pure Chemicals (Tokyo, Japan). Terminal deoxynucleotidyl transferase (TdTase) was obtained from TOYOBO (Osaka, Japan). The Envision kit was from Dako Co (Carpinteria, CA). Biotin

14-ATP and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen (Carlsbad, CA). VECTASHIELD was from Vector Laboratories (Burlingame, CA). HilyMax and 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) were from Dojindo Laboratories (Kumamoto, Japan). The RNeasy kit and HiPerFect were obtained from Qiagen (Valencia, CA), the PrimeScript® 1st strand cDNA Synthesis Kit was purchased from TAKARA Bio (Ohtsu, Japan), and iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). Antibodies against CHOP, actin and GRP78 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz,CA) and that against CD68 was from Dack Co (Carpinteria, CA). Alpha-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN) was from Alexis (San Diego, CA). HCT-15 and RAW264 cells were obtained from the Cell Resource Center for Biochemical Research at Tohoku University (Sendai, Japan) and RIKEN BioResource Center (Tsukuba, Japan), respectively. CHOP-null mice that had been backcrossed with wild-type mice (C57BL/6) for more than 10 times and the wild-type mice (5-7-weeks-old, male) were prepared and there was no apparent phenotypes in CHOP-null mice as described previously.[24] The experiments and procedures described here were carried out in accordance with the Guide for the Care and

Use of Laboratory Animals as adopted and promulgated by the National Institute of Health, and were approved by the Animal Care Committee of Kumamoto University.

Development of DSS- or TNBS-induced Colitis and Measurement of Colon Length and Disease Activity Index (DAI)

DSS-induced colitis was induced in mice by the addition of 3% DSS (w/v, final concentration) to their drinking water as described previously.[23] The animals were allowed free access to the DSS-containing water for 7 days. For histopathological observation, measurement of MPO, various mRNAs, ROS, TBARS as well as apoptosis, we used rectum and distal colon.

After 7 days, animals were placed under deep ether anaesthesia and sacrificed, the colons were dissected and their length measured from the ileocecal junction to the anal verge.

The DAI was determined macroscopically by an observer unaware of the treatment the mice had received, according to previously reported criteria.[23,25] Briefly, the DAI was calculated as the sum of the diarrheal stool score (0: normal stool; 1: mildly

soft stool; 2: very soft stool; 3: watery stool) and the bloody stool score (0: normal color stool; 1: brown color stool; 2: reddish color stool; 3: bloody stool).

TNBS-induced colitis was produced by intrarectal administration of TNBS once as described previously.[26]

Myeloperoxidase (MPO) Activity

MPO activity in the colonic tissues was measured as previously described.[23,27] After DSS-treatment, colons were dissected, rinsed with cold saline and cut into small pieces. Samples were homogenized and protein concentrations of the supernatants were determined using the Bradford method.[28] MPO activity was determined in 10 mM phosphate buffer with 0.5 mM *o*-dianidisine, 0.00005% (w/v) hydrogen peroxide and 20 μg protein. MPO activity was obtained from the slope of the reaction curve and its specific activity was expressed as the number of hydrogen peroxide molecules converted per min per mg protein.

Lipid Peroxidation Measured by Thiobarbituric Acid Reactant Substances (TBARS)

The amounts of TBARS in colonic tissues were measured as previously described.[29-31] After DSS-treatment, colons were dissected, cut into small pieces and weighed. Samples were homogenized and centrifuged. Supernatants were mixed with 20 µl of 8.1% SDS solution, 150 µl of 20% acetic acid solution and 5 µl of 0.8% BHT solution, then with 150 µl of 0.8% TBA solution, and finally boiled for 1 h. Samples were mixed with 500 µl of *n*-butanol/pyridine (15:1) and centrifuged. The absorbance of the supernatant was measured at 532 nm and the amount of TBARS expressed as the number of TBARS molecules per gram of tissue.

Immunoblotting Analysis

Total protein was extracted from the colonic tissues as described previously.[23,32] The protein concentration of the samples was determined by the Bradford method.[28] Samples were applied to 8% (GRP78 and actin) or 12% (CHOP) polyacrylamide SDS

gels and subjected to electrophoresis, after which the proteins were immunoblotted with appropriate antibodies.

Real-time RT-PCR Analysis

This was done as described[33] with some modifications. Total RNA was extracted from intestinal tissues using an RNeasy kit according to the manufacturer's protocol. Samples (2.5 μg RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument (Bio-Rad)) experiments using iQ SYBR GREEN Supermix and analyzed with Opticon Monitor Software. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal standard.

Primers were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used were

(name: forward primer, reverse primer): For mouse; *Tnf-α*: 5'-cgtcagccgatttgctatct-3', 5'-cggactccgcaaagtctaag-3'; *Il-1β*: 5'-gatcccaagcaatacccaaa-3', 5'-ggggaactctgcagactcaa-3'; *Il-6*: 5'-ctggagtcacagaaggagtgg-3', 5'-ggtttgccgagtagatctcaa-3'; *Vcam-1* (vascular cell adhesion molecule): 5'-ctcctgcacttgtggaaatg-3', 5'-tgtacgagccatccacagac-3'; *Icam-1*: 5'-tcgtgatggcagcctcttat-3', 5'-gggcttgtcccttgagtttt-3'; *Madcam-1* (mucosal addressin cell adhesion molecule): 5'-gcaggctgggagctactct-3', 5'-tccctcttgtggtaggttgc-3'; *CD49d*: 5'-cagagccacacccaaaagtt-3', 5'-tgaaatgtcgtttgggtcttt-3'; *CD11b*: 5'-tgtgagcagcactgagatcc-3', 5'-atggctccactttggtctct-3'; *L-selectin*: 5'-attcctgtagccgtcatggt-3', 5'-catccttttcttgagatttcttgc-3'; *Il-10*: 5'-ggccctttgctatggtgtcc-3', 5'-aagcggctgggggatgac-3'; *Caspase-11*: 5'-tggaagctgatgctgtcaag-3', 5'-gagcctcctgttttgtctcg-3'; endoplasmic reticulum oxidoreductin (*Ero*)-*1α*: 5'-ttaagtctgcgagctacaagtattc-3', 5'-agtaagtccacatactcagcatcg-3'; *Bcl-2*: 5'-cctgtggatgactgagtacc-3', 5'-gagacagccaggagaaat-3'; *Chop*: 5'-acagaggtcacacgcacatc-3', 5'-gggcactgaccactctgttt-3'; *Grp78*: 5'-gcttccgataatcagccaac-3', 5'- gcaggaggaattccagtca-3'; *C/ebp-β*:

5'-gcaagagccgcgacaag-3', 5'-ggctcgggcagctgctt-3'. For human; *CHOP*: 5'-tgcctttctcttcggacact-3', 5'-tgtgacctctgctggttctg-3'.

Histological and Immunohistochemical Analysis

Colonic tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned.

For histological examination (hematoxylin and eosin (H & E) staining), sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. Samples were mounted with Malinol and inspected with the aid of an Olympus BX51 microscope. For histologic evaluation of the tissue damage (damage score) and areas of lesions (extent of lesion), sections were evaluated microscopically by an observer unaware of the treatment the animals had received, and quantified as described.[34,35] Colonic damage (damage score) was categorized into 6 groups (0: normal mucosa; 1: infiltration of inflammatory cells; 2: shortening of the crypt by less than 50%; 3: shortening of the crypt by more than 50%; 4: crypt loss; 5: destruction of epithelial cells).

The extent of lesions in the total colon was categorized into 6 grades (0: 0%; 1: 1%–20%; 2: 21%–40%; 3: 41%–60%; 4: 61%–80%; 5: 81%–100%).

For immunohistochemical analysis, sections were treated in a microwave oven with 0.01 M citric acid buffer for antigen activation and incubated with 0.3% hydrogen peroxide in methanol for removal of endogenous peroxidase. Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with each antibody in the presence of 3% BSA and then incubated for 1 h with peroxidase-labelled polymer conjugated to goat anti-rabbit immunoglobulins (Envision kit). Then, 3, 3'-diaminobenzidine (DAB) was applied to the sections and the sections were finally incubated with Mayer's hematoxylin. Samples were mounted with malinol and inspected using a fluorescence microscope (Olympus BX51).

Overexpression and Suppression of Expression of Targeting Genes

The CHOP- and ERO-1α-specific siRNAs were purchased from Qiagen. A plasmid expressing CHOP or C/EBP-β was as described.[36,37] HCT-15 and RAW264 cells were transfected with these siRNAs or plasmids using HiPerFect or HilyMax

(transfection reagents) according to the manufacturer's instructions. Non-silencing siRNA (5'-uucuccgaacgugucacgudTdT-3' and 5'-acgugacacguucggagaadTdT-3') was used as a negative control.

Preparation of Mouse Peritoneal Macrophages

Mouse peritoneal macrophages were prepared as described previously.[23,38] Mice were given 0.5 ml of 10% proteose peptone by intraperitoneal injection and peritoneal cells were harvested 3 days later. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. After incubation for 4 h, non-adherent cells were removed and the adherent cells were cultured for use in the experiments. Virtually all of the adherent cells were macrophages, as previously described.[38] Caspase-1 activity was measured as described.[39] The amounts of IL-1β secreted into the medium were measured by ELISA according to the manufacturer's protocol.

TdT-mediated dUTP-biotin End Labelling (TUNEL) Assay