Genetic Evidence for a Protective Role for Heat Shock Factor 1 and Heat Shock Protein 70 against Colitis*

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Inflammatory bowel disease (IBD) involves infiltration of leukocytes into intestinal tissue, resulting in intestinal damage induced by reactive oxygen species (ROS). Pro-inflammatory cytokines and cell adhesion molecules (CAMs) play important roles in this infiltration of leukocytes. The roles of heat shock factor 1 (HSF1) and heat shock proteins (HSPs) in the development of IBD are unclear. In this study, we examined the roles of HSF1 and HSPs in an animal model of IBD, dextran sulfate sodium (DSS)-induced colitis. The colitis worsened or was ameliorated in HSF1-null mice or transgenic mice expressing HSP70, respectively. Administration of DSS up-regulated the expression of HSP70 in colonic tissues in an HSF1-dependent manner. Expression of pro-inflammatory cytokines and CAMs and the level of cell death observed in colonic tissues were increased or decreased in DSS-treated HSF1-null mice or transgenic mice expressing HSP70, respectively, relative to control wild-type mice. Relative to macrophages from control wild-type mice, macrophages prepared from HSF1-null mice or transgenic mice expressing HSP70 displayed enhanced or reduced activity, respectively, for the generation of pro-inflammatory cytokines in response to lipopolysaccharide stimulation. Suppression of HSF1 or HSP70 expression in vitro stimulated lipopolysaccharide-induced up-regulation of CAMs or ROS-induced cell death, respectively. This study provides the first genetic evidence that HSF1 and HSP70 play a role in protecting against DSS-induced colitis. Furthermore, this protective role seems to involve various mechanisms, such as suppression of expression of pro-inflammatory cytokines and CAMs and ROS-induced cell death.

Inflammatory bowel disease (IBD),2 Crohn disease, and ulcerative colitis have become substantial health problems with an actual prevalence of 200–500 cases/100,000 people in western countries, which almost double every 10 years (1). Although the etiology of IBD is not yet fully understood, recent studies suggest that IBD involves chronic inflammatory disorders in the intestine because of “a vicious cycle.” Infiltration into intestinal tissues causes intestinal mucosal damage induced by reactive oxygen species (ROS) that are released from the activated leukocytes, and this intestinal mucosal damage further stimulates the infiltration of leukocytes (2). To understand the molecular mechanism underlying the pathogenesis of IBD and to develop new types of clinical drugs for IBD, identification of endogenous factors that positively or negatively affect the development of IBD is important. For this purpose, various experimental animal colitis models, in particular the dextran sulfate sodium (DSS)- and trinitrobenzenesulfonic acid-induced colitis models, have been used (3).

Pro-inflammatory cytokines play an important role in the activation and infiltration of leukocytes that are associated with IBD. This conclusion is supported by a range of evidence. Increases in the levels of various pro-inflammatory cytokines (such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6) in intestinal tissues has been reported in both IBD patients and animal models of IBD (4, 5). TNF-α-deficient mice or transgenic mice with enhanced production of TNF-α show a phenotype of resistance to trinitrobenzenesulfonic acid-induced colitis or the spontaneous development of an IBD-like disorder, respectively (6, 7). A chimeric monoclonal antibody against TNF-α, infliximab, is effective for the treatment of Crohn disease patients (6, 8). Because TNF-α secondarily stimulates the production of many other pro-inflammatory cytokines (9), TNF-α is thought to play a crucial role in the pathogenesis of IBD.

Cell adhesion molecules (CAMs) also play an important role in the infiltration of leukocytes associated with IBD, as is suggested by the following evidence. CAMs expressed on vascular endothelial cells (such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)) bind to counterparts expressed on leukocytes, and this binding is an essential step in the recruitment of blood circulating leukocytes into inflamed tissues (10). Up-regulation of the expression of various CAMs, including VCAM-1 and ICAM-1, in intestinal tissues in both IBD patients and animal models of IBD has been reported (10, 11). Immune neutralization of VCAM-1 significantly sup-

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2 The abbreviations used are: IBD, inflammatory bowel disease; ROS, reactive oxygen species; DSS, dextran sulfate sodium; TNF-α, tumor necrosis factor-α; IL, interleukin; CAMs, cell adhesion molecules; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; HSPs, heat shock proteins; HSF1, heat shock factor 1; LPS, lipopolysaccharide; DAI, disease activity index; TBARS, thiobarbituric acid-reactive substance(s); VLA-4, very late antigen-4; MadCAM-1, mucosal addressin cell adhesion molecule-1; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyltransferase-mediated biotinylated UTP nick end labeling.
presses trinitrobenzenesulfonic acid-induced colitis (12), whereas ICAM-1–deficient mice show a phenotype of resistance to DSS–induced colitis (13).

Heat shock proteins (HSPs) are induced by various stressors (such as ROS) to provide cellular resistance to these stressors (14–16). This up-regulation of HSP expression by stressors is achieved at the level of transcription through a consensus cis-element (heat shock element) and a transcription factor (heat shock factor 1 (HSF1)) that specifically binds to a heat shock element located upstream of the hsp genes (17). An essential role for HSF1 in the stressor–up-regulation of HSPs was demonstrated by the observation that disruption to the activity of HSF1 leads to the loss of stressor–induced HSP up-regulation (17, 18). HSPs and HSF1 also have attracted considerable attention as candidates for endogenous factors that affect the development of IBD because some HSPs, including HSP70, were reported to be overexpressed in the intestinal tissues of IBD patients and in an animal model of IBD (19–23). HSF1 negatively regulates the expression of TNF-α and IL-1β genes (24, 25). The expression of copper/zinc superoxide dismutase-1, which is protective against IBD, is positively regulated by HSF1 (26, 27). All these previous results suggest that HSPs and HSF1 have negative roles in the development of IBD (i.e. protective roles against IBD); however, there is no direct evidence (such as genetic evidence) to support this idea. Furthermore, there are some data that suggest positive roles for HSPs and HSF1 in the development of IBD: HSF1 positively regulates expression of the IL-6 gene; extracellular HSPs elicit an inflammatory response; and autoantibodies and reactive T–cells against HSPs have been found in IBD patients (28–31). Therefore, the effects of genetic alteration of HSPs and HSF1 on the development of colitis in animal models of IBD should be examined to understand the exact role (positive or negative) of HSPs and HSF1 in IBD. In this study, we used HSF1–null mice and transgenic mice expressing HSF1, and their respective wild-type mice (8–10 weeks old) were prepared as described previously (31, 32). Transgenic mice expressing HSP70 and their wild-type counterparts (8–10 weeks old) were gifts from Drs. C. E. Angelidis and G. N. Pagoulatos (University of Ioannina, Greece) and were prepared as described previously (33). Homozygotic transgenic mice expressing HSP70 and heterozygotic transgenic mice expressing HSF1 were used in experiments.

Development of DSS–induced Colitis and Measurement of Colon Length and the Disease Activity Index (DAI)—Colitis was induced in mice by the addition of 3% (w/v) DSS (final concentration) to their drinking water. The animals were allowed free access to the DSS-containing water for 7 days. After 7 days, the animals were placed under deep ether anesthesia and killed; the colons were dissected and 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 (34). Briefly, the DAI was calculated as the sum of the diarrheal stool score (0, normal stool; 1, mildly soft stool; 2, very soft stool; and 3, watery stool) and the bloody stool score (0, normal color stool; 1, brown color stool; 2, redish color stool; and 3, bloody stool).

Myeloperoxidase Activity—Myeloperoxidase activity in the colonic tissues was measured as described previously (35, 36). After 7 days of DSS treatment, animals were placed under deep ether anesthesia and killed. Colons were dissected, rinsed with cold saline, and cut into small pieces. Samples were homogenized in 50 mM phosphate buffer, freeze-thawed, and centrifuged. The protein concentrations of the supernatants were determined using the Bradford method (37). Myeloperoxidase activity was determined in 10 mM phosphate buffer with 0.5 mM o-dianisidine, 0.00005% (w/v) hydrogen peroxide, and 20 μg of protein. Myeloperoxidase 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/mg of protein.
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Lipid Peroxidation Measured by Thiobarbituric Acid-reactive Substances (TBARS)—The amounts of TBARS in the colonic tissues were measured as described previously (35, 38). After 7 days of DSS treatment, animals were placed under deep ether anesthesia and killed. Colonos were dissected, rinsed with cold saline, cut into small pieces, and weighed. Samples were homogenized in 1.15% KCl solution 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% butylated hydroxytoluene solution; shaken vigorously for 1 min; mixed with 150 μl of 0.8% thiobarbituric acid solution; shaken vigorously for 1 min; and finally boiled for 1 h. Samples were mixed with 500 μl of 1-butanol and pyridine (15:1), shaken vigorously for 1 min, and centrifuged. The absorbance of the supernatant was measured at 532 nm, and the amount of TBARS was expressed as the number of TBARS molecules/g of tissue.

Real-time Reverse Transcription-PCR Analysis—Total RNA was extracted from colonic tissues using the RNeasy kit according to the manufacturer’s protocol. Samples (2.5 μg of RNA) were reverse-transcribed using the first-strand cDNA synthesis kit according to the manufacturer’s instructions. Synthesized cDNA was used in real-time reverse transcription-PCR (Chromo 4 system, Bio-Rad) experiments using iQ SYBR Green Supermix and analyzed with Opticon Monitor software according to the manufacturer’s instructions. The real-time PCR cycle conditions were 50 °C for 2 min, followed by 90 °C for 10 min, and finally 45 cycles at 95 °C for 30 s and at 63 °C for 60 s. 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 or actin cDNA was used as an internal standard.

Primers were designed using the Primer3 website (frodo.wi.mit.edu/cgi-bin/ primer3/primer3 www.cgi). The primers used for detection of mouse cDNA included the following: *hsp25*, 5′-ctcttcctcttcctccctgag-3′ (forward) and 5′-ttggctccagctgctgca-3′ (reverse); *hsf1*, 5′-cctcctgctgctgctgca-3′ (forward) and 5′-ctcctgctgctgctgca-3′ (reverse); and *hsf1*, 5′-cctcctgctgctgcaacttt-3′ (forward) and 5′-tgcctgtctcgagtt-3′ (reverse). The primers used for detection of human cDNA included the following: *HSP70, 5′-aggccacaagatcactc-3′* (forward) and 5′-ttgcctctttgttactc-3′ (reverse); and *HSF1, 5′-gaaagtcccagcctagtc-3′* (forward) and 5′-cctcagctgtcagtt-3′ (reverse).

Histological and Immunohistochemical Analyses—Colonic tissue samples were fixed in 4% buffered paraformaldehyde, embedded in optimal cutting temperature compound, and cryosectioned. For histological examination (hematoxylin and eosin staining), sections were stained first with Mayer’s hematoxylin and then with 1% eosin alcohol solution. Samples were mounted with malinol and inspected using an Olympus IX70 or BX51 microscope. For histological 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 (39, 40). Colonic damage was categorized into 6 groups (0, normal mucosa; 1, infiltration of inflammatory cells; 2, shortening of the crypt by <50%; 3, shortening of the crypt by >50%; 4, crypt loss; and 5, destruction of epithelial cells (ulceration and erosion)). The extent of lesions in the total colon was categorized into six grades (0, 0%; 1, 1–20%; 2, 21–40%; 3, 41–60%; 4, 61–80%; and 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 antibody against *HSP70* (1:200 dilution) in the presence of 2.5% bovine serum albumin, and then incubated for 1 h with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins. 3,3′-Diaminobenzidine was applied to the sections, which were then incubated with Mayer’s hematoxylin. Samples were mounted with malinol and inspected using an Olympus IX70 or BX51 fluorescence microscope.

Small Interfering RNA (siRNA) Targeting of Genes—The HSF1- and *HSP70*-specific siRNAs were purchased from Qiagen Inc. HCT-15 and bEnd.3 cells were transfected with siRNA using HiPerFect transfection reagent according to the manufacturer’s instructions. Non-silencing siRNA (5′-uucuuccgacguacacguacTdT-3′ and 5′-acugacugacuacgagaTdT-3′) was used as a negative control.

Preparation of Mouse Peritoneal Macrophages and Enzyme-linked Immunosorbent Assay—Mouse peritoneal macrophages were prepared as described previously (41). Mice were given 2 ml of 10% protease peptone by intraperitoneal injection, and peritoneal cells were harvested 3 days later. The cells were seeded in 35-mm culture dishes at 1 × 10⁶ cells/dish in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. After incubation for 4 h, non-adherent cells were removed, and adherent cells were cultured for use in the experiments. Virtually all of the adherent cells were macrophages, as described previously (41). The amounts of pro-inflammatory cytokines secreted into the medium were measured by enzyme-linked immunosorbent assay according to the manufacturer’s protocol.
Terminal Deoxynucleotidyltransferase-mediated Biotinylated UTP Nick End Labeling (TUNEL) Assay—Colonic tissue samples were fixed in 4% buffered paraformaldehyde, embedded in optimal cutting temperature compound, and cryosectioned. Sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37 °C, then with terminal nucleotidyltransferase and biotin-14-ATP for 1 h at 37 °C, and finally with Alexa Fluor 488-conjugated streptavidin and 4',6-diamidino-2-phenylindole (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected using an Olympus IX70 or BX51 fluorescence microscope.

Statistical Analysis—All values are expressed as the mean ± S.E. Two-way analysis of variance, followed by Scheffe’s multiple comparison test or Tukey’s test, was used to evaluate differences between groups. Student’s t test for unpaired results was used to evaluate differences between two groups. Differences were considered to be significant for p values <0.05.

RESULTS

DSS-induced Colitis and Expression of HSPs in HSF1-null Mice—The severity of DSS-induced colitis can be monitored by various indexes, such as body weight, DAI, colon length, myeloperoxidase activity, TBARS amount, and histological indexes. We compared the time course of development of colitis induced by 3% DSS administration in HSF1-null mice and wild-type mice (ICR) by monitoring body weight and the DAI. Administration of 3% DSS caused a mild increase in the DAI, but did not affect the body weight of the wild-type mice. In contrast, administration of 3% DSS resulted in a higher DAI score and loss of body weight in HSF1-null mice (Fig. 1, A and B). DSS-induced colon shortening, used as a morphometric measure for the degree of inflammation, was more severe in HSF1-null mice than in wild-type mice (Fig. 1, C). DSS-induced colon shortening was measured daily. After 7 days, colon length (C), colonic myeloperoxidase (MPO) activity (D), and colonic TBARS (E) were determined as described under “Experimental Procedures.” After 7 days, sections of colonic tissues were prepared and subjected to histological examination (hematoxylin and eosin staining), and the damage score and extent of lesion for eight independent sections were determined (G and H). One of the sections is shown (F). Values are the mean ± S.E. (n = 3–10). *, p < 0.05; **, p < 0.01; n.s., not significant.
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Type mice (Fig. 1C). Colonic myeloperoxidase activity, an indicator of leukocyte infiltration, was much higher in DSS-administered HSF1-null mice than in wild-type mice (Fig. 1D). Colonic TBARS, an index of lipid peroxidation associated with inflammation, was also higher in DSS-administered HSF1-null mice than in wild-type mice (Fig. 1E). Fig. 1F shows the results of histological analysis of colonic tissues prepared from DSS-administered and untreated HSF1-null and wild-type mice. More extensive crypt loss, epithelial destruction, and leukocyte infiltration were observed in sections from DSS-administered HSF1-null mice than in those from wild-type mice. Histological score analysis revealed that the histological differences were statistically significant (Fig. 1, G and H). The results in Fig. 1 show that HSF1-null mice are more sensitive to DSS-induced colitis than their wild-type counterparts.

We monitored the expression of hsp mRNAs in the colonic tissues of DSS-administered and untreated HSF1-null and wild-type mice by real-time reverse transcription-PCR. The expression of hsp70 (but not hsp25 or hsp60) mRNA was significantly lower in DSS-treated HSF1-null mice than in wild-type mice (Fig. 2A). DSS administration clearly up-regulated the expression of hsp70 mRNA (Fig. 2A). On the basis of these results, we subsequently focused on HSP70.

Immunohistochemical analysis demonstrated that DSS administration increased the levels of HSP70 in colonic mucosa and around colonic vessels in wild-type mice, but not in HSF1-null mice (Fig. 2B). Fig. 2 (C and D) shows the protein level of HSP70 as assessed by the immunoblotting assay. The level of HSP70 was clearly lower in DSS-administered HSF1-null mice than in wild-type mice. The results also show that DSS administration did not clearly increase the amount of HSP70, which is inconsistent with the results in Fig. 2B. This may be because total colonic tissues or only damaged tissues were used for immunoblotting or immunohistochemical analysis, respectively.

On the basis of the results in Fig. 2, we consider that the inability of HSF1-null mice to induce synthesis of HSP70 is responsible for their phenotypic sensitivity to DSS-induced colitis (Fig. 1).

DSS-induced Colitis in Transgenic Mice Expressing HSF1 or HSP70—The development of DSS-induced colitis in transgenic mice expressing HSF1 and wild-type mice (C57/BL6) was compared (Fig. 3). As shown in Fig. 3 (A and B), trans-
FIGURE 3. Development of DSS-induced colitis in transgenic mice expressing HSF1 and wild-type mice. Development of DSS-induced colitis was induced and monitored in transgenic mice expressing HSF1 (HSF1 Tg) and wild-type mice (WT; C57/BL6) as described in the legend of Fig. 1 (A–E). Values are the mean ± S.E. (n = 3–5). MPO, myeloperoxidase. *, p < 0.05; ***, p < 0.001; n.s., not significant. The expression of HSP70 in colonic tissues was monitored by immunohistochemical analysis as described in the legend of Fig. 2 (F).
genic mice expressing HSF1 were more resistant than wild-type mice to DSS-dependent loss of body weight and increase in the DAI. The differences in the extent of DSS-induced colitis in the two groups of wild-type mice (shown in Figs. 1 and 3) must be due to their different genetic backgrounds (ICR and C57/BL6). Judging from other indexes of colitis (colon length, colonic myeloperoxidase activity and colonic TBARS), it is obvious that transgenic mice expressing HSF1 developed less DSS-induced colitis than their wild-type counterparts (Fig. 3, C–E). Immunohistochemical analysis demonstrated that HSP70 staining was more obvious in the colonic tissues of transgenic mice compared with wild-type mice with or without DSS treatment (Fig. 3F). This implies that the higher expression of HSP70 in the transgenic mouse relative to the wild-type mouse is responsible for its phenotype of resistance to DSS-induced colitis. The tissue section in Fig. 3F shows significant colitis even in transgenic mice expressing HSF1. This is because induction of HSP70 is clear around lesions; thus, we present the section prepared from tissues around lesions. When we performed histological score analysis (as in Fig. 1, G and H), the results showed that both colonic damage and the extent of lesions were lower in transgenic mice expressing HSF1 than in wild-type mice (data not shown).

To test this idea, the development of DSS-induced colitis was compared in transgenic mice expressing HSP70 and their wild-type counterparts (C57/BL6) (Fig. 4). Although there was no clear difference in DSS-dependent loss of body weight in the two groups (Fig. 4A), a DSS-dependent increase in the DAI was clearly suppressed in transgenic mice expressing HSP70 compared with wild-type mice (Fig. 4B). All of the other indexes of colitis that were tested (colon length, colonic myeloperoxidase activity, and...
colonic TBARS) showed that transgenic mice expressing HSP70 were more resistant than wild-type mice to DSS-induced colitis (Fig. 4, C–E). By immunohistochemical analysis, we confirmed that HSP70 expression was much higher in the colonic tissues of transgenic mice than in those of wild-type mice regardless of whether or not they were treated with DSS (Fig. 4F). The results in Fig. 4 suggest that HSP70 expression somehow suppresses DSS-induced colitis.

Involvement of Cytokines in Alteration of DSS-induced Colitis in HSF1-null Mice and Transgenic Mice Expressing HSP70—To understand the mechanism governing the increased susceptibility of HSF1-null mice to DSS-induced colitis, we compared the mRNA expression of various inflammation-related proteins in the colonic tissues. As shown in Fig. 5A, the mRNA expression of TNF-α, IL-1β, and IL-6 was much higher in DSS-treated HSF1-null mice than in wild-type mice. On the other hand, the mRNA expression of superoxide dismutase-1 was indistinguishable between HSF1-null and wild-type mice even after DSS administration (Fig. 5A). These results are consistent with the idea that the higher levels of mRNA expression of these pro-inflammatory cytokines in the colonic mucosa of HSF1-null mice are responsible for their increased susceptibility to DSS-induced colitis.

The mRNA expression of these pro-inflammatory cytokines was also compared in transgenic mice expressing HSP70 and wild-type mice. As shown in Fig. 5B, the mRNA expression of TNF-α, IL-1β, and IL-6 in colonic tissues was significantly lower in DSS-treated transgenic mice expressing HSP70 than in wild-type mice. These results are consistent with the idea that the lower mRNA expression of these pro-inflammatory cytokines in DSS-treated transgenic mice expressing HSP70 is responsible for their DSS-induced colitis resistance phenotype.

The results in Fig. 5 suggest that HSF1 and HSP70 negatively regulate the expression of the selected pro-inflammatory cytokines under inflammatory conditions. To test this idea in vitro, we compared the LPS-stimulated production of the pro-inflammatory cytokines
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(TNF-α, IL-1β, and IL-6) in peritoneal macrophages prepared from transgenic mice and their wild-type counterparts. As shown in Fig. 6A, LPS stimulated the production of all of these pro-inflammatory cytokines. The level of TNF-α was much higher in the medium of HSF1-null macrophages than in that of wild-type macrophages after LPS treatment; however, there was no significant difference in the levels of IL-1β and IL-6 between the two groups (Fig. 6A). These results suggest that HSF1 is involved in the production of TNF-α (but not IL-1β and IL-6) under inflammatory conditions.

In contrast, the levels of not only TNF-α but also of IL-1β and IL-6 were much lower in the medium of LPS-treated macrophages prepared from transgenic mice expressing HSP70 compared with wild-type mice. These results suggest that the expression of HSP70 may suppress the production of these pro-inflammatory cytokines under inflammatory conditions.

Involvement of CAMs in Altering the Susceptibility of HSF1-null Mouse and Transgenic Mice Expressing HSP70 to DSS-induced Colitis—CAMs can be divided into two groups: those expressed mainly on vascular endothelial cells (such as VCAM-1, ICAM-1, and MadCAM-1) and those expressed mainly on leukocytes (such as VLA-4, Mac-1, and L-selectin) (10). As shown in Fig. 5A, the mRNA expression of vcam-1, icam-1, and madcam-1 in colonic tissues was higher in HSF1-null mice than in wild-type mice. In contrast, differences in the mRNA expression of vla-4, mac-1, and L-selectin in colonic tissues were not statistically significant between HSF1-null mice and their wild-type counterparts (Fig. 5A).

We also compared the mRNA expression of vcam-1, icam-1 and madcam-1 in the colonic tissues of transgenic mice expressing HSP70 and their wild-type counterparts. The mRNA expression of these CAMs was much lower in DSS-administered transgenic mice than in wild-type mice (Fig. 5B).

The results in Fig. 5 suggest that the expression of CAMs is negatively regulated by HSF1 and HSP70 under inflammatory conditions. To test this idea in vitro, we examined the effect of siRNA specific for HSF1 or HSP70 on the LPS-induced mRNA expression of the CAMs in bEnd.3 cells. Transfection with siRNA specific for HSF1 clearly inhibited the mRNA expression of hsf1 in both the presence and absence of LPS (Fig. 7A). Transfection with HSF1 siRNA up-regulated the mRNA expression of vcam-1 and icam-1 but down-regulated that of madcam-1 in the presence of LPS (Fig. 7A). These results suggest that HSF1 negatively regulates the mRNA expression of vcam-1 and icam-1 (but not madcam-1) under inflammatory conditions.

Transfection of the cells with siRNA specific for HSP70 inhibited the mRNA expression of hsp70 in both the presence and absence of LPS. However, it did not significantly up-regulate the mRNA expression of the CAMs (but instead down-regulated the mRNA expression of vcam-1) in the presence of LPS, suggesting that, at least in vitro, HSP70 does not negatively regulate the mRNA expression of these CAMs under inflammatory conditions.

Involve of ROS-induced Cell Death in Altering DSS-induced Colitis in HSF1-null Mice and Transgenic Mice Expressing HSP70—We compared the level of cell death in the colonic mucosa of DSS-administered HSF1-null mice or transgenic mice expressing HSP70 and the respective wild-type mice using the TUNEL assay. More TUNEL-positive cells (cell death) were observed in the colonic mucosa of DSS-administered HSF1-null mice or transgenic mice expressing HSP70 and the respective wild-type mice (Fig. 8A). On the other hand, fewer TUNEL-positive cells were observed in the colonic mucosa of DSS-administered transgenic mice expressing HSP70 than in that of wild-type mice (Fig. 8B). The results suggest that ROS-induced cell death associated with DSS-induced colitis is stimulated or suppressed in HSF1-null mice or transgenic mice expressing HSP70, respectively.

To test the role of HSF1 and HSP70 in ROS-induced cell death in vitro, we examined the effect of siRNA specific for...
of cell death in colonic mucosa and the improved resistance to DSS-induced colitis observed in transgenic mice expressing HSP70.

**DISCUSSION**

A number of reports using HSF1-null mice and/or transgenic mice expressing HSP70 have shown that HSF1-dependent induction of HSP expression is protective against the development of various diseases, such as gastric ulcers, heart failure, pancreatitis, hypoxic/ischemic brain injury, and spinal and bulbar muscular atrophy (42–46). Because some HSPs have been reported to be overexpressed in the intestinal tissues of IBD patients (19, 20, 23) and in an animal model of IBD (21–23), HSF1 and HSPs are thought to be involved in the pathogenesis of IBD. However, as was described in the Introduction, some studies have suggested that these proteins inhibit and others have suggested that they promote the development of IBD. In this study, we have gathered evidence that HSF1 and HSPs have negative roles in the development of IBD (protective roles against IBD) by demonstrating the sensitive phenotype of HSF1-null mice and the resistant phenotype of transgenic mice expressing HSP70 (or HSF1) against DSS-induced colitis, an animal model for IBD. Proposed mechanisms for positive roles of HSF1 and HSP70 in the development of IBD (such as immunoactivation by extracellular HSPs) may be present; however, these effects may be masked by the protective roles of HSF1 and HSP70 against DSS-induced colitis (see below). This study provides the first genetic evidence for involvement of HSF1 and HSPs in IBD-related colitis. Furthermore, we have examined the molecular mechanisms governing the susceptibility of HSF1-null mice and transgenic mice expressing HSP70 to DSS-induced colitis, focusing on the expression of pro-inflammatory cytokines and CAMs and ROS-induced cell death both in vivo and in vitro (see below).

Pro-inflammatory cytokines (in particular, TNF-α) positively contribute to the progression of IBD and colitis in animal models of IBD (6, 7). DSS-induced mRNA expression of various pro-inflammatory cytokines (TNF-α, IL-1β, and

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**FIGURE 7. Effect of siRNAs for HSF1 and HSP70 on the mRNA expression of various CAMs.** bEnd.3 cells were transfected with 1.2 μg of siRNA for HSF1 (siHSF1; A), siRNA for HSP70 (siHSP70; B), or non-silencing siRNA (ns; A and B). After 24 h, cells were incubated with or without 5 μg/ml LPS for 18 h. The relative mRNA expression of each gene was monitored and is expressed as described in the legend of Fig. 2. Values are the mean ± S.E. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant.

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HSF1 or HSP70 on cell death induced by menadione, a superoxide anion (a representative ROS)-releasing drug, in a colon cancer cell line (HCT-15). Transfection of these cells with siRNA for HSF1 clearly inhibited the mRNA expression of hsf1 in both the presence and absence of menadione (Fig. 9A). As shown in Fig. 9B, treatment of cells with menadione induced cell death in a dose-dependent manner. Transfection of cells with HSF1 siRNA did not clearly affect menadione-induced cell death. (A slight stimulation of cell death was observed with 50 μM menadione.) On the other hand, transfection of cells with siRNA for HSP70 inhibited the mRNA expression of hsp70 in both the presence and absence of menadione (Fig. 9C) and clearly stimulated cell death induced by menadione, but did not affect cell viability in the absence of menadione (Fig. 9D). The results in Fig. 9 suggest that HSP70 protects colonic cells from ROS-induced cell death and that this effect may be involved in the lower level
ICAM-1, ICAM-2, and ICAM-3 in colonic tissues was stimulated or inhibited in HSF-null mice or transgenic mice expressing HSP70, respectively. We consider that this stimulation or inhibition of cytokine mRNA expression is responsible for the DSS-induced colitis phenotypes exhibited by these mice. Because the observation that deficiency of HSF1 stimulates the production of TNF-α but not IL-1β and IL-6 was reproduced in vitro (in the model of LPS-induced production of pro-inflammatory cytokines in peritoneal macrophages), HSF1 seems to be directly involved in the expression of TNF-α, but not IL-1β and IL-6. HSF1 suppresses the mRNA expression of TNF-α through binding to the heat shock element located in the promoter of TNF-α as described previously. Because NF-κB positively regulates the expression of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Furthermore, it is also known that up-regulation of HSP70 expression by heat shock inhibits the inflammatory stimuli-dependent activation of NF-κB through various mechanisms.

CAMs also positively contribute to the progression of IBD and colitis in animal models of IBD through recruitment of blood circulating leukocytes into inflamed intestinal tissues. DSS administration induced the mRNA expression of CAMs expressed mainly on vascular endothelial cells and leukocytes. However, HSF1 deficiency affected only the mRNA expression of CAMs on vascular endothelial cells, suggesting that these CAMs rather than those on leukocytes contribute to the greater sensitivity of HSF-null mice to DSS-induced colitis relative to control wild-type mice. The DSS-induced mRNA expression of icam-1, vcam-1, and madcam-1 was suppressed in transgenic mice expressing HSP70, and this may be involved in conferring resistance to DSS-induced colitis. In vitro, transfection with HSF1 siRNA stimulated the LPS-induced mRNA expression of icam-1 and vcam-1, suggesting that HSF1 negatively regulates the mRNA expression of icam-1 and vcam-1, which seems to be responsible for the higher levels of mRNA expression of these CAMs in HSF1-null mice.
in vivo. On the other hand, HSF1 siRNA did not stimulate the LPS-induced mRNA expression of madcam-1, and HSP70 siRNA did not stimulate the LPS-induced mRNA expression of all of these CAMs. These observations suggest that the alterations to the mRNA expression of these CAMs seen in vivo are achieved indirectly, for example, through up-regulation of TNF-α, which has been reported to induce the expression of these CAMs both in vivo and in vitro (10).

Colonic mucosal cell death induced by ROS released from activated leukocytes is thought to be directly responsible for the pathogenesis of human IBD and DSS-induced colitis (2). Analysis using the TUNEL assay revealed that cell death in colonic mucosa was stimulated or inhibited in HSF1-null mice or transgenic mice expressing HSP70, respectively. This correlates with other parameters for DSS-induced colitis; however, it was not clear whether these alterations to cell death cause or result from the progression of DSS-induced colitis. Given that transfection with siRNA for HSP70 stimulated ROS-induced cell death in vitro, this result suggests that HSP70 protects colonic mucosal cells from ROS-induced cell death, which seems to contribute to the lower level of cell death seen in the colonic mucosa of DSS-administered transgenic mice expressing HSP70. On the other hand, transfection with siRNA specific for HSF1 did not stimulate ROS-induced cell death in vitro to the same extent. Thus, the higher level of cell death seen in the colonic mucosa of DSS-administered HSF1-null mice is the result (rather than the cause) of aggravation of DSS-induced colitis.

The results of this study suggest that nontoxic inducers of HSP expression are therapeutically beneficial for IBD. Supporting this notion, geranylgeranylacetone (a leading anti-ulcer drug in the Japanese market and a nontoxic HSP inducer) (53) suppresses both DSS- and trinitrobenzenesulfonic acid-induced colitis (54, 55). However, the ability of geranylgeranylatedon to induce HSP expression is not strong, which may explain its relatively weak effect on these types of colitis (54, 55). Therefore, we propose that more potent nontoxic HSP inducers would be therapeutically beneficial for IBD.

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