especially that of HSP70, provides resistance as they re-fold or degrade denatured proteins produced by the stressors (Beere et al., 2000; Gething and Sambrook, 1992; Jaattela, 1999; Jaattela et al., 1998; Kiang and Tsokos, 1998; Mathew and Morimoto, 1998; Ravagnan et al., 2001; Saleh et al., 2000). It has been reported not only that various gastric irritants, including ethanol, up-regulate HSPs, but also that artificial up-regulation of HSPs confers resistance to these irritants in cultured gastric mucosal cells (Hirakawa et al., 1996; Mizushima et al., 1999; Nakamura et al., 1991; Saika et al., 2000; Tomisato et al., 2000; Tomisato et al., 2001). Similar up-regulation of HSPs by gastric irritants has also been recorded *in vivo*, in addition to which whole body heat treatment has been shown to suppress gastric irritant-induced lesions (Itoh and Noguchi, 2000; Otani et al., 1997; Saika et al., 2000; Zeniya et al., 1995). Although these findings strongly indicate that HSPs are protective, very little direct evidence exists, and to date no *in vivo* study has been conducted to demonstrate that inhibition of HSPs results in a phenotype susceptible to irritant-induced gastric lesions.

Interestingly, geranylgeranylacetone (GGA), a leading anti-ulcer drug on the Japanese market, has been reported to be a non-toxic HSP-inducer, up-regulating

various HSPs not only in cultured gastric mucosal cells at concentrations that do not affect cell viability but also in various tissues, including the gastric mucosa *in vivo* (Hirakawa et al., 1996; Katsuno et al., 2005; Ooie et al., 2001; Yasuda et al., 2005). We have previously reported that pre-induction of HSPs by GGA protects cultured gastric mucosal cells from cell death induced by various irritants, including ethanol, hydrochloric acid, hydrogen peroxide and NSAIDs (Mizushima et al., 1999; Takano et al., 2002; Tomisato et al., 2000; Tomisato et al., 2001). These previous results suggest that the anti-ulcer effect of GGA is due to its HSP-inducing activity. However, because GGA mediates various other gastro-protective mechanisms, such as an increase in GMBF, stimulation of surface mucus production and direct protection of gastric mucosal cell membranes (Kunisaki and Sugiyama, 1992; Terano et al., 1986; Ushijima et al., 2005), it remains unclear whether up-regulation of HSPs represents GGA's major mode of anti-ulcer activity.

The up-regulation of HSPs by various stressors including heat shock is regulated at the transcription level by a consensus *cis*-element (heat shock element (HSE)) and a transcription factor (heat shock factor 1 (HSF1)), that specifically binds to HSE located

on the upstream region of *hsp* genes (Morimoto, 1998). The essential role of HSF1 in the up-regulation of HSPs, conferring cytoprotection against stressors, was demonstrated by the observation that disruption of the activity of HSF1 leads to the loss of stressor-induced HSP up-regulation and the emergence of cells that are sensitive to apoptosis (McMillan et al., 1998; Morimoto, 1998). Furthermore, analysis of HSF1-null mice revealed that up-regulation of HSPs is involved in various physiological and pathological phenomena, suggesting that HSF1-null mice provide a powerful tool for examination of the role of HSPs *in vivo* (Christians et al., 2000; Xiao et al., 1999; Yan et al., 2002). In this study, we used the HSF1-null mouse model to obtain direct genetic evidence for the contribution of HSPs to the protection of the gastric mucosa. We also investigated whether up-regulation of HSPs by GGA contributes to its anti-ulcer activity.

Materials and Methods

Chemicals and Animals. Paraformaldehyde, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and PGE_2 were obtained from Sigma. PGE_2 ELISA kit was from Cayman Chemical. Antibodies against HSP25, HSP60, HSP70, HSP90 or actin were purchased from Stressgen or Santa Cruz Biotechnology. Optimal cutting temperature (OCT) compound was from Sakura Fintechnical. Mayer's hematoxylin, 1% eosin alcohol solution and Malinol were from MUTO Pure Chemicals. Terminal deoxynucleotidyl transferase (TdTase) was obtained from TOYOBO. Biotin 14-ATP, Alexa Fluor 488 (or 594) goat anti-rabbit immunoglobulin G and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen. VECTASHIELD was from Vector Laboratories. 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) was from Dojindo Co. The RNeasy kit was obtained from Qiagen, the first-strand cDNA synthesis kit from Amersham, and SYBR GREEN PCR Master Mix from ABI. HSF1-null and wild-type mice (ICR) were prepared as described previously (Inouye et al., 2004) and both of mice of 10 – 12 weeks and 25 – 30 g were used in experiments.

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.

Gastric Damage Assay. Gastric ulcerogenic response was examined as described previously (Tomisato et al., 2004), with some modifications. Mice, which had been fasted for 24 h, were orally administered either ethanol or hydrochloric acid (5 ml/kg). Four hours later, the animals were sacrificed with an overdose of ether, after which their stomachs were removed and scored for hemorrhagic damage by an observer unaware of the treatment they had received. Calculation of the scores involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index. Gastric mucosal PGE_2 levels were determined by ELISA as previously described (Futaki et al., 1993).

Cell Culture, over-expression and SiRNA targeting of HSP70. Human gastric carcinoma (AGS) cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO_2 at 37°C. Cells were exposed to ethanol by changing the medium. Cells were cultured for 24 h and used in experiments. Cell viability was determined by the MTT method. The transfection with pcDNA3.1 containing the *hsp70* gene (Fujimoto et al., 2005) was carried out using Lipofectamine (TM2000) according to the manufacturer's instructions. Cells were used for experiments after a 18 h recovery period.

We used siRNA of 5'-ggagcuggagcaggugugudTdT-3' and 5'-acacaccugcuccagcuccdTdT-3' as annealed oligonucleotides for repressing HSP70 expression. AGS cells were transfected with siRNA using RNAiFect transfection reagent according to the manufacturer's instructions. Non-silencing siRNA (5'-uucuccgaacgugucacgudTdT-3' and 5'-acgugacacguucggagaadTdT-3') was used as a negative control.

Real-time RT-PCR Analysis. Total RNA was extracted from the gastric mucosa using an RNeasy kit according to the manufacturer's protocols. Samples (10 μg RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA (15 ng) was applied to real-time RT-PCR (ABI PRISM 7700) using SYBR GREEN PCR Master Mix and analysed with ABI PRISM 7700 Sequence Detection Software according to the manufacturer's instructions. Real-time cycle conditions were 2 min at 50°C, followed by 10 min at 90°C and finally 45 cycles at 95°C for 30 s and 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, the gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

Primers were designed using the Primer3 Web site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers are listed as follows: gene name: forward primer, reverse primer. *hsp25*: 5'-cctcttccctatcccctgag-3', 5'-ttggctccagactgttcaga-3'; *hsp60*: 5'-cgttgccaataacacaaacg-3', 5'-cttcaggggttgtcacaggt-3'; *hsp70*: 5'-

tggtgctgacgaagatgaag-3', 5'-aggtcgaagatgagcacgtt-3'; *hsp90*■: 5'-aaaggcagaggctgacaaga-3', 5'-aggggaggcatttcttcagt-3'; *hsp90*■: 5'-gcggcaaagacaagaaaaag-3', 5'-gaagtggtcctcccagtcat-3'.

Immuno-Blotting Analysis. Total protein was extracted from the gastric mucosa as described previously (Tsutsumi et al., 2002). The protein concentration of the samples was determined by the Bradford method. Samples were applied to 8% (HSP70 and HSP90), 10% (HSP60 and actin) or 12% (HSP25) polyacrylamide SDS gels and subjected to electrophoresis, after which the proteins were immuno-blotted with appropriate antibodies.

Histological and Immunohistochemical Analysis. Gastric tissue samples were fixed in 4% buffered paraformaldehyde, embedded in OCT compound and cryosectioned. Sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution for histological examination (Hematoxylin and eosin (HE) staining). Samples were mounted with Malinol and inspected using an Olympus IX70 microscope.

For immunohistochemical analysis, 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% BSA, and finally incubated for 1 h with Alexa Fluor 488 (or 594) goat anti-mouse immunoglobulin G in the presence of DAPI (5 μg/ml). Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX70).

TdT-mediated dUTP-biotin end labelling (TUNEL) Assay. Gastric tissue samples were fixed in 4% buffered paraformaldehyde, embedded in OCT compound and cryosectioned. Sections were incubated first with protenase K (10 μg/ml) for 15 min at 37°C, then with TdTase and biotin 14-ATP for 1 h at 37°C and finally with Alexa Fluor 488 conjugated with streptavidin for 1 h. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX70).

Measurement of Gastric Acid Secretion and GMBF. Gastric acid secretion was measured as previously described (Filaretova et al., 2002), with some modifications.

Under ether anaesthesia, the abdomen was opened and the pylorus was ligated. After drug administration, mice were sacrificed and their stomachs were removed. The gastric contents were collected and titrated with 10 mM NaOH to pH 7.0 using an automatic titrator (TITRONIC basis, SCHOTT).

GMBF was measured as described elsewhere (Takeuchi et al., 2003), with some modifications. Under urethane-anaesthetized conditions, the stomach was exposed and mounted in an *ex vivo* chamber. GMBF was measured with a laser Doppler flowmeter (ALF-21, Adavence).

Statistical Analysis. All values are expressed as the mean ± standard error (S.E.M.). Two-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test or Tukey test was used for evaluation of differences between groups. The Student's *t*-test for unpaired results was used for the evaluation of differences between two groups. Differences were considered to be significant for values of $P<0.05$.

Results

Enhanced Gastric Ulcerogenic Response. The development of gastric lesions following oral administration of ethanol was compared between wild-type and HSF1-null mice. Exposure to ethanol produced gastric lesions in a dose-dependent manner (Fig. 1A). As shown in Fig. 1B, intragastric administration of 40% ethanol resulted in significant gastric lesions in HSF1-null mice but not in wild-type mice. Lack of the *hsf1* gene did not significantly affect the background level (without ethanol administration) of production of gastric lesions (Fig. 1B). These results show that HSF1 plays an important role in protecting the gastric mucosa from ethanol-induced lesions. We also examined the prevalence of hydrochloric acid-induced gastric lesions in HSF1-null mice, revealing that oral administration of 0.6 N hydrochloric acid produces more severe lesions in HSF1-null mice than in wild-type mice (Fig. 1C). Therefore, the protective effects of HSF1 do not appear to be mediated in response to a specific stressor, such as ethanol.

Ethanol-induced Up-regulation of HSPs in Gastric Mucosa. Given that HSF1 up-regulates the expression of HSPs at the transcriptional level, we examined the effect of ethanol administration on the expression of *hsp* mRNAs and HSPs in the gastric mucosa of HSF1-null mice and wild-type mice. Figure 2A shows the level of various *hsp* mRNAs that were detected by real-time RT-PCR. Lack of the *hsf1* gene did not affect the background level of expression of *hsp* mRNAs, as reported previously (Inouye et al., 2003; McMillan et al., 1998; Xiao et al., 1999). Ethanol administration up-regulated the level of *hsp25, hsp70* and *hsp90*■ but not *hsp60* and *hsp90* mRNA in wild-type mice (Fig. 2A). This up-regulation was not observed in HSF1-null mice (Fig. 2A). Among *hsp25, hsp70* and *hsp90*■ mRNAs, the *hsp70* mRNA displayed the strongest HSF1-dependent up-regulation induced by ethanol (Fig. 2A).

Figure 2B shows the protein level of various HSPs as assessed by the immuno-blotting assay. Unlike the results of the mRNA analysis (Fig. 2A), lack of the *hsf1* gene caused a decrease in the background expression level of HSP25, HSP60, HSP70 and HSP90 (Fig. 2B). This is the first examination of the background expression level of

HSPs in stomach of HSF1-null mice and results were consistent with previous data in other organs and cell species of HSF1-null mice, such as liver, fibroblasts and dendritic cells (Xiao et al., 1999; Zheng and Li, 2004). Ethanol administration up-regulated the production of only HSP70, a response that was dependent on the function of HSF1 (Fig. 2B). Being different from results in mRNA level (Fig. 2A), the protein level of HSP25 and HSP90 was not up-regulated by the ethanol administration (Fig. 2B) and we have no clear explanation for this discrepancy at present. Based on the results illustrated in Fig. 2, together with those of a previous study which suggested that, among the HSPs, HSP70 plays a major role in cytoprotection (Beere et al., 2000; Gething and Sambrook, 1992; Jaattela, 1999; Jaattela et al., 1998; Mathew and Morimoto, 1998; Ravagnan et al., 2001; Saleh et al., 2000), we subsequently focused on HSP70.

In order to examine the ethanol-dependent up-regulation of HSP70 in the gastric mucosa in detail, we performed histological and immunohistochemical analyses. Sections were prepared from the gastric tissues of HSF1-null and wild-type mice that had been exposed to ethanol. HE and DAPI staining shows the presence of gastric mucosal lesions in both HSF1-null mice (with both 40% and 100% ethanol

administration) and wild-type mice (with only 100% ethanol administration) (Fig. 3), this being consistent with the results illustrated in Fig. 1B. Furthermore, immunohistochemical analysis with an antibody against HSP70 demonstrated that HSP70 is induced by administration of 100% ethanol in wild-type mice, and that this up-regulation is most apparent in the vicinity of gastric lesions (Fig. 3A), suggesting that HSP70 induced by ethanol play an important role in development of gastric lesions. In contrast, no significant up-regulation of HSP70 was observed in HSF1-null mice following administration of either 40% or 100% ethanol (Fig. 3B). This pattern of HSP70 expression is consistent with the results illustrated in Fig. 2B. Together, the results outlined in Figs. 2 and 3 show that the induction HSP70 in the gastric mucosa following oral administration of ethanol is dependent on HSF1 function.

Mechanism for Stimulated Production of Gastric Lesions in HSF1-null Mice. In order to investigate the mechanism governing the severity of production of ethanol-stimulated gastric lesions in HSF1-null mice, we compared various factors that are known to be important for the production of gastric lesions (including the level of

apoptosis, gastric acid secretion, GMBF and the level of PGE_2) between HSF1-null and wild-type mice. Figure 4 illustrates the level of gastric mucosal apoptosis as determined by TUNEL assay. In wild-type mice, an increase in TUNEL-positive (apoptotic) cells was observed following the administration of 100% but not 40% ethanol, whereas a clear increase in TUNEL-positive cells was observed with 40% ethanol administration in the HSF1-null mice (Fig. 4). Similar level of TUNEL-positive cells was observed following the administration of 100% ethanol in the HSF1-null mice (data not shown). Lack of the *hsf1* gene did not affect the background level of TUNEL-positive cells (Fig. 4). These results show that induction of apoptosis by ethanol is enhanced in HSF1-null mice compared to wild-type mice; in other words, HSF1 protects gastric mucosal cells from ethanol-induced apoptosis.

We also examined the role of HSP70 in ethanol-induced cell death *in vitro*, using over-expression plasmid and siRNA for HSP70. Transfection of AGS cells with the plasmid containing *hsp70* gene caused over-expression of HSP70 in both absence and presence of 7% ethanol (Fig. 5A). This transfection made cells resistant to cell death induced by 7% ethanol (Fig. 5B). On the other hand, transfection of siRNA for

the *hsp70* decreased the expression of HSP70 in both absence and presence of 7% ethanol (Fig. 5C) and made cells sensitive to cell death induced by 7% ethanol (Fig. 5D). These results suggest that HSP70 protect gastric cells against ethanol-induced cell death.

Gastric acid secretion is also an important factor affecting the production of lesions, representing another potential aggressive insult on the gastric mucosa. We therefore examined the effect of ethanol on gastric acid secretion in wild-type and HSF1-null mice. As shown in Fig. 6A, gastric acid secretion was increased by the addition of histamine, as described previously (Furutani et al., 2003). Administration of 40% ethanol did not affect gastric acid secretion in either wild-type or HSF1-null mice (Fig. 6B). Similar results were obtained with 100% ethanol (data not shown). Furthermore, both the background level of gastric acid secretion and that recorded after administration of 40% ethanol were not significantly affected by the lack of the *hsf1* gene (Fig. 6B). These results suggest that the stimulation of ethanol-induced gastric lesion production in HSF1-null mice does not involve a change in gastric acid secretion.

GMBF is another important factor in the production of gastric lesions, with a decrease in GMBF having a causative effect. As shown in Fig. 6C, GMBF in wild-type

mice was stimulated by the addition of PGE_2, as described previously (Araki et al., 2000). However, no significant difference in GMBF was recorded between wild-type and HSF1-null mice (Fig. 6C), indicating that HSF1 does not affect GMBF.

As described above, PGE_2 is a major defensive factor for the gastric mucosa, exerting a protective effect against various irritants by decreasing gastric acid secretion, and increasing GMBF, in addition to other mechanisms (Miller, 1983). Therefore, as the results illustrated in Fig. 6A-C suggest, it is unlikely that aggravation of ethanol-induced gastric lesions in HSF1-null mice involves PGE_2. In order to confirm this, we examined the effect of ethanol on gastric mucosal PGE_2 levels in wild-type and HSF1-null mice. As shown in Fig. 6D, ethanol administration did not affect the level of PGE_2 in either group of animals. Furthermore, there was no significant difference in PGE_2 levels between wild-type and HSF1-null mice in either the presence or absence of ethanol treatment (Fig. 6D). These results support the idea that stimulation of ethanol-induced gastric lesions in HSF1-null is not attributable to the impairment of PGE_2 production.

Anti-ulcer and HSP-inducing Activities of GGA in HSF1-null Mice. In order to evaluate the contribution of the HSP-inducing activity of GGA to its anti-ulcer activity, we investigated the effect of GGA in HSF1-null mice. First, we examined the effect of GGA and/or ethanol on gastric mucosal HSP70 expression in wild-type mice, revealing a potent expression induced by ethanol, and a lower level of expression in response to GGA (Fig. 7A). Interestingly, pre-administration of GGA enhanced the ethanol-dependent HSP70 response (Fig. 7A). Figure 7C shows the effect of pre-administration of GGA on ethanol-produced gastric lesions in wild-type and HSF1-null mice. In order to obtain similar levels of gastric lesions, 100% and 40% ethanol administration were administered to wild-type and HSF1-null mice, respectively. In fact, 40% ethanol administration in HSF1-null mice caused the comparable lesion score as 100% ethanol administration in wild-type mice (Fig. 7C). Pre-administration of GGA significantly suppressed the ethanol-dependent production of gastric lesions in wild-type mice (Fig. 7C), as described previously (Murakami et al., 1981). In contrast, no significant effect was recorded in the HSF1-null mice (Fig. 7C). We confirmed that administration of

GGA and/or 40% ethanol did not induce HSP70 (Fig. 7B). This result shows that HSF1 is required for the efficacy of the anti-ulcer activity of GGA against ethanol. Overall, the results in Fig. 7 suggest that the loss of the protective effect of GGA in HSF1-null mice is due to the lack of expression of HSPs (such as HSP70); in other words, the HSP-inducing activity of GGA contributes to its anti-ulcer activity.

Discussion

A number of previous observations have suggested that HSPs and their up-regulation by gastric irritants play an important role in protecting the gastric mucosa against lesion development. Artificial up-regulation of HSPs, especially HSP70, by GGA (a clinically used anti-ulcer drug) or other methods in cultured gastric mucosal cells confers protection from irritant-induced cell death (Hirakawa et al., 1996; Mizushima et al., 1999; Nakamura et al., 1991; Takano et al., 2002; Tomisato et al., 2000; Tomisato et al., 2001), while exposure to such irritants induces HSP production (Itoh and Noguchi, 2000; Otani et al., 1997; Saika et al., 2000; Zeniya et al., 1995). In this study, we found that HSF1-null mice are more susceptible to irritant-induced gastric lesions, providing direct genetic evidence for the significance of HSPs in ameliorating the outcome of irritant-induced gastric insults. Further genetic evidence in support of this notion has recently been published, revealing that transgenic mice over-expressing human HSP27 display a phenotype that is resistant to NSAID-induced gastric lesions (Ebert et al., 2005).