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Stimulation of gastric ulcer healing by heat shock protein 70

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ABSTRACT

It is important in treatment of gastric ulcers to not only prevent further ulcer formation but also enhance ulcer healing. When cells are exposed to gastric irritants, expression of heat shock proteins (HSPs) is induced, making the cells resistant to the irritants. We recently reported direct evidence that HSPs, especially HSP70, are preventive against irritant-induced gastric ulcer formation. Gastric ulcer healing is a process involving cell proliferation and migration at the gastric ulcer margin and angiogenesis in granulation tissue. In this study, we have examined the role of HSP70 in gastric ulcer healing. Gastric ulcers were produced by focal and serosal application of acetic acid. Expression of HSP70 was induced in both the gastric ulcer margin and granulation tissue. Compared with wild-type mice, gastric ulcer healing was accelerated in transgenic mice expressing HSP70, and both cell proliferation at the gastric ulcer margin and angiogenesis in granulation tissue were enhanced. Oral administration of geranylgeranylacetone, an inducer of HSPs, to wild-type mice, either prior to or after ulcer formation, not only induced expression of HSP70 in the stomach but also accelerated gastric ulcer healing. On the other hand, oral administration of purified recombinant HSP70 prior to the ulcer formation, but not after formation, stimulated gastric ulcer healing. This study provides the first evidence that HSP70 accelerates gastric ulcer healing. The results also suggest that both the HSP70 produced prior to ulcer formation and released from damaged cells, and the HSP70 produced after ulcer formation are involved in this accelerated healing process.

1. Introduction

The balance between aggressive and defensive factors determines the development of gastric lesions, with either a relative increase in aggressive factors or a relative decrease in defensive factors resulting in lesions. The gastric mucosa is challenged by a variety of both endogenous and exogenous irritants (aggressive factors), including ethanol, gastric acid, pepsin, reactive oxygen species, non-steroidal anti-inflammatory drugs (NSAIDs) and *Helicobacter pylori* [1]. In order to protect the gastric mucosa, a complex defence system, which includes the production of surface mucus (gastric mucin) and bicarbonate and the regulation of gastric mucosal blood flow has evolved. Prostaglandins (PGs), in particular PGE_2, enhance these protective mechanisms, and are therefore thought to be major gastric defensive factors [2].

Recently, heat shock proteins (HSPs) have also attracted considerable attention as major gastric defensive factors. When cells are exposed to stressors, HSPs are induced in a manner that is dependent on a transcription factor, heat shock factor 1 (HSF1). The up-regulation of HSPs, especially that of HSP70, provides resistance to such stressors given that intracellular HSPs re-fold or degrade denatured proteins produced by the stressors [3,4]. We recently reported that HSF1-null mice or transgenic mice expressing HSP70 show sensitive or resistant phenotypes, respectively, to irritant-induced gastric lesions [5,6], providing genetic evidence that HSPs, especially HSP70, play important roles in the protection of gastric mucosa from irritant-induced lesion formation. Interestingly, geranylgeranylacetone (GGA), one of the standard anti-ulcer drugs on the Japanese market, has been reported to be an HSP-inducer, up-regulating HSPs not only in cultured gastric mucosal cells but also at the gastric mucosa [7–10]. We recently showed that the HSP-inducing activity of GGA mainly contributes to its gastro-protective activity against ethanol and NSAIDs [5,6]. In these experiments, we used 50–200 mg/kg doses of GGA by oral administration 1 h before the administration of ethanol or NSAIDs and observed the ulcer formation 4 h or 8 h after the administration of ethanol or NSAIDs, respectively [5,6].

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; PGs, prostaglandins; HSPs, heat shock proteins; HSF1, heat shock factor 1; GGA, geranylgeranylacetone; bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; BrdU, 5-bromo-2′-deoxyuridine; EIA, enzyme immuno assay; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAMP, damage-associated molecular patterns.

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HSP70 has also been detected in extracellular compartments and the actions of extracellular HSP70 have recently been paid much attention. It has been reported that HSP70 could be released from cells through both passive (leaked from necrotic cells) and active (released by exocytosis) routes [11,12]. Such extracellular HSP70 binds to high-affinity receptors, including toll-like receptors, to induce the innate immune response [13–16]. Although extracellular HSP70 should be present at the gastric mucosa, especially when ulcerated, the role of extracellular HSP70 at this site is unknown.

Gastric ulcer healing is a complex process that includes inflammatory response (such as an increase in the level of PGE_2), re-epithelialization due to cell proliferation and migration at the gastric ulcer margin and angiogenesis in granulation tissue [17–20]. Expression of growth factors such as basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF) is induced by inflammatory responses and they activate epithelial cell migration and proliferation at the gastric ulcer margin and angiogenesis in granulation tissue to enhance ulcer healing [17,21–23].

For the effective treatment of gastric ulcers, not only the prevention of further ulcer formation, but also the enhancement of ulcer healing is important. However, no data have been reported for the role of HSP70 in gastric ulcer healing. In this study, we have examined the role of HSP70 in gastric ulcer healing, using transgenic mice expressing HSP70 and in response to treatment with GGA. The results suggest that expression of HSP70 accelerates gastric ulcer healing by increasing the level of PGE_2 and the expression of growth factors, thereby stimulating cell proliferation at the gastric ulcer margin and angiogenesis in granulation tissue. The results also suggest that both intracellular and extracellular HSP70 are involved in this acceleration.

2. Materials and methods

2.1. Chemicals and animals

GGA was a gift from Eisai (Tokyo, Japan). Formaldehyde, bovine serum albumin (BSA) and 5-bromo-2′-deoxyuridine (BrdU) were obtained from Sigma (St. Louis, MO). A PGE_2 enzyme immuno assay (EIA) kit was purchased from Cayman Chemical (Ann Arbor, MI). Quercetin was obtained from Wako Pure Chemical Industries (Osaka, Japan). An enzyme-linked immunosorbent assay (ELISA) kit for mouse VEGF and an antibody against HSP70 (for immunoblotting analysis) were from R&D Systems (Minneapolis, MN). An antibody against HSP70 (for immunohistochemical analysis) was obtained from Stressgen (Ann Arbor, MI, USA). Antibodies against actin and BrdU were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against CD31, biotinylated anti-rat immunoglobulins and streptavidin-HRP were from BD Biosciences (San Jose, CA). Mayer's hematoxylin and malinol were from MUTO Pure Chemicals (Tokyo, Japan). The RNeasy kit was obtained from QIAGEN (Valencia, CA), the first-strand cDNA synthesis kit was from Takara (Kyoto, Japan), and iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). Transgenic mice expressing HSP70 and their wild-type counterparts (C57/BL6) were gifts from Drs. C.E. Angelidis and G.N. Pagoulatos (University of Ioannina, Ioannina, Greece) and were prepared (6–8 weeks of age and 20–25 g) as described previously [24]. Homozygotic male transgenic mice expressing HSP70 were used in these experiments. The experiments and procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committee of Keio University.

2.2. Development of gastric ulcers

Gastric ulcers were produced by exposure of tissue to acetic acid according to a previously described method [25]. In brief, under ether anaesthesia, the abdomen was incised and the stomach exposed. A round plastic mold (4 mm in diameter) was placed on the serosal surface of the corpus and acetic acid (40%; 100 μl) was poured into the mold to treat the surface for 10 s. The treated surface was rinsed with saline, the abdomen was closed and the animals were routinely maintained. Control mice were operated in the same manner as the experimental group but not exposed to the acetic acid.

GGA (10 ml/kg as an emulsion with 5% gum arabic) was orally administered once only at day 0 (2 h before ulcer formation) once daily from day 3 to day 6 or day 8 (the ulcer was induced at day 0). We used 200 mg/kg doses of GGA, because this dose of GGA was shown to induce the expression of HSP70 clearly on our previous reports [5,6].

For measurement of gastric lesions, 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 millimetres squared and summing the values to give an overall gastric lesion index.

Gastric mucosal PGE_2 level was determined by EIA, as previously described [26]. The amount of VEGF in gastric tissue was measured by ELISA according to the manufacturer's protocol. For labeling with BrdU, BrdU (100 mg/kg) was injected intraperitoneally, 1 h before the mice were sacrificed, as described previously [27].

2.3. Real-time RT-PCR analysis

Total RNA was extracted from gastric tissue using an RNeasy kit according to the manufacturer's protocol. Samples (2.5 μg of 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 (Bio-Rad Chromo 4 system) 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 95 °C for 3 min, followed by 44 cycles at 95 °C for 10 s and 60 °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 (GAPDH) cDNA was used as an internal standard.

Primers were designed using the Primer3 website. The primers used were (name, forward primer and reverse primer): bFGF: 5′-cccgacggccgcgtggat-3′, 5′-acttagaagccagcagccg-3′; IGF, 5′-gctggaccagagacccttg-3′, 5′-gctccggaagcaacactca-3′; TGF-β1, 5′-tgacgtcactggagtacgg-3′, 5′-ggttcatgtcatggatggtgc-3′; GAPDH, 5′-aactttggcattgtggaagg-3′ and 5′-acacattgggggtaggaaca-3′.

2.4. Immunohistochemical analysis

Gastric tissue samples were fixed in 10% buffered formalin and embedded in paraffin before being cut into 4 μm-thick sections.

For immunohistochemical analysis for HSP70 and BrdU, sections were incubated with 0.3% hydrogen peroxide in methanol for removal of endogenous peroxidase. For detection of BrdU, sections were treated in a microwave oven with 0.01 M citric acid buffer (pH 6.0) for antigen activation before the incubation with hydrogen peroxide. Sections were blocked with 3% BSA for 30 min,

incubated for 12 h with antibody against HSP70 (1:200 dilution) or BrdU (1:100 dilution) in the presence of 2.5% BSA, 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 incubated with Mayer's hematoxylin. Samples were mounted with malinol and inspected with the aid of a microscope (Olympus BX51).

For immunohistochemical analysis for CD31, sections were incubated with 0.3% hydrogen peroxide in methanol and then incubated with 20 μg/ml proteinase K for 20 min for antigen activation before blocking with 3% BSA for 30 min. Sections were incubated for 12 h with antibody against CD31 (1:50 dilution) in the presence of 2.5% BSA and then for 30 min with biotinylated anti-rat immunoglobulins. Sections were incubated for 30 min with streptavidin-HRP, following which 3,3′-diaminobenzidine was applied and the sections were finally incubated with Mayer's hematoxylin. Samples were mounted with malinol and inspected with the aid of a fluorescence microscope (Olympus BX51).

2.5. Immunoblotting analysis

Whole cell extracts were prepared as described previously [28]. The protein concentration of the sample was determined by the Bradford method [29]. Samples were applied to polyacrylamide SDS gels and subjected to electrophoresis, and the resultant proteins were immunoblotted with each antibody.

2.6. Purification of recombinant HSP70

The purification of His-tagged protein was performed as described previously [30]. The pET21 plasmid containing *hsp70* was introduced into *Escherichia coli* (BL21) cells and HSP70 was overproduced by incubation with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 30 °C. Cells were lysed by digestion with lysozyme in buffer A (50 mM NaH_2PO_4 (pH 8.0) and 0.5 M NaCl) containing 2 μg/ml pepstatin A, 1 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride, and centrifuged. The supernatant was subjected to Ni-NTA agarose (Sigma) column chromatography, and HSP70 was eluted with buffer A containing 250 mM imidazole.

2.7. Statistical analysis

All values are expressed as the mean ± S.E.M. Two-way ANOVA followed by the Tukey test or a Student's *t* test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

3. Results

3.1. Alteration of gastric expression of HSP70 during ulcer healing

Changes in the gastric expression of HSP70 were examined for an acetic acid-induced ulcer during the healing process. The lesion

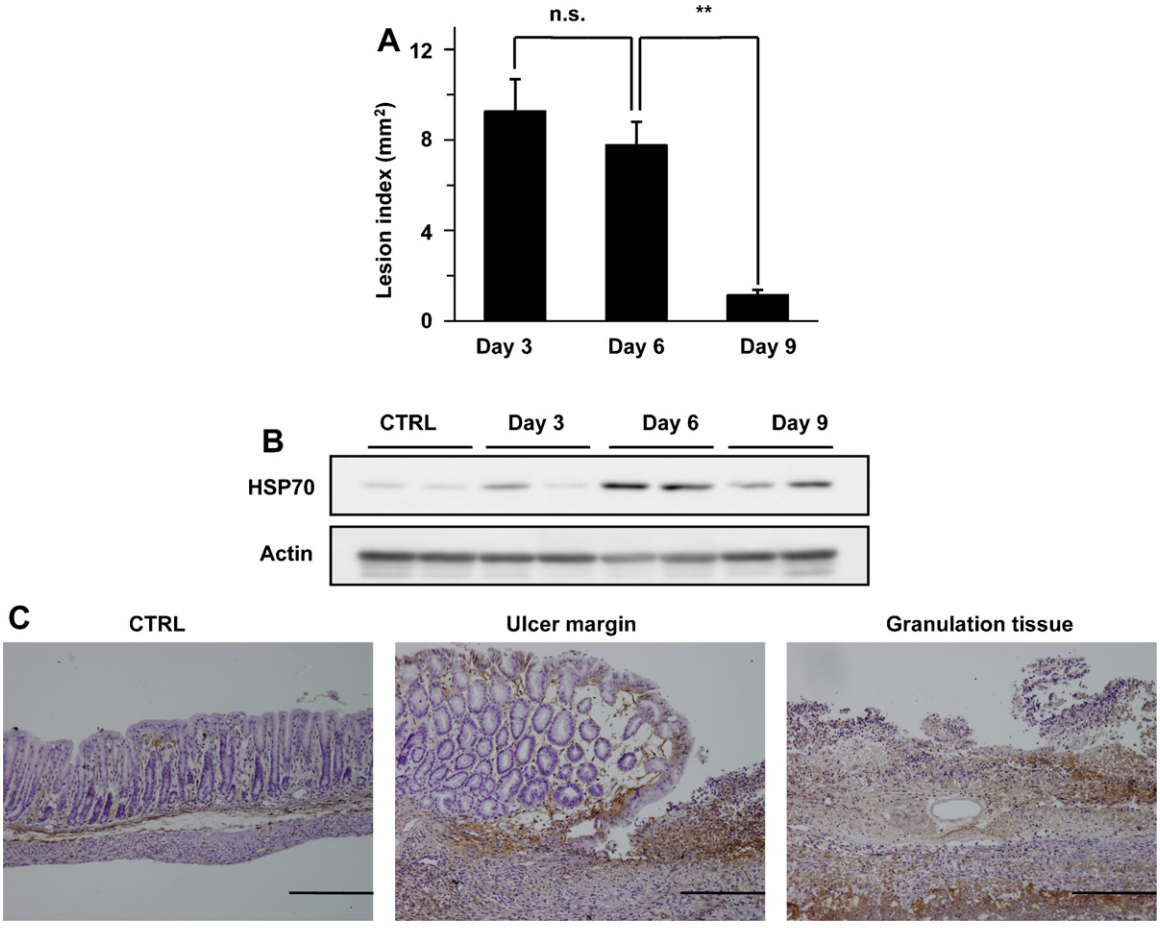

Fig. 1. Expression of HSP70 during gastric ulcer healing. Gastric ulcers were induced in wild-type mice by exposure of the luminal side of their stomachs to acetic acid at day 0, as described in Section 2, and the stomachs were removed at day 3, 6 or 9. Normal stomachs (without ulcer induction) (CTRL) were also prepared, as described in Section 2 (A–C). The stomachs were scored for hemorrhagic damage (A). Whole cell extracts were prepared from the stomachs and analyzed by immunoblotting with an antibody against HSP70 or actin (B). Sections of gastric tissues prepared at day 6 were subjected to immunohistochemical analysis with an antibody against HSP70 (C). Values are mean ± S.E.M. (n = 3–5) $^{**}P < 0.01$; n.s., not significant. Scale bar, 200 μm.

index decreased from day 3 to day 9 (Fig. 1A), showing that gastric ulcer healing progresses in this period. Immunoblotting analysis revealed that the expression of HSP70 was induced during this period of gastric ulcer healing (Fig. 1B). Immunohistochemical analysis with antibody against HSP70 revealed that induction of expression of HSP70 was observed both at the ulcer margin and in granulation tissue (Fig. 1C), suggesting that induced HSP70 plays an important role in gastric ulcer healing.

3.2. Effect of expression of HSP70 on gastric ulcer healing

In order to understand the role of HSP70 in gastric ulcer healing, we compared the progression of gastric ulcer healing in transgenic mice expressing HSP70 and in wild-type mice. As shown in Fig. 2A, the decrease in the lesion index after the development of a gastric ulcer was more rapid in the transgenic mice than in the wild-type mice. By immunoblotting analysis, we confirmed that HSP70 was expressed at high levels in the stomach in both control transgenic mice and in transgenic mice in which ulcers had been induced (Fig. 2B and C). These results suggest that expression of HSP70 accelerates gastric ulcer healing.

Cell proliferation at the gastric ulcer margin is important for gastric ulcer healing. To examine the effect of expression of HSP70 on cell proliferation at the gastric ulcer margin, we compared the number of BrdU-positive cells (proliferating cells) in transgenic mice expressing HSP70 and in wild-type mice by immunohistochemical analysis. The number of BrdU-positive cells at the gastric ulcer margin was higher in transgenic mice expressing HSP70 than in wild-type mice (Fig. 2D and E), suggesting that expression of HSP70 stimulates cell proliferation at the gastric ulcer margin. On the other hand, the background level of cell proliferation, that is in the absence of ulcer development, was indistinguishable between the wild-type and transgenic mice (Fig. 2D and E).

Angiogenesis in granulation tissue is also important for gastric ulcer healing. To examine the effect of expression of HSP70 on angiogenesis in granulation tissue, we compared the number of vessels by monitoring the expression of CD31, a marker for vascular endothelial cells between transgenic mice expressing HSP70 and in wild-type mice. In both types of mice, the number of vessels was higher in granulation tissue than in normal tissue (from mice without gastric ulcers), confirming that there was a higher level of angiogenesis in the granulation tissue (Fig. 2F and G). Furthermore, the number of vessels in the granulation tissue was higher in transgenic mice expressing HSP70 than in wild-type mice (Fig. 2F and G), suggesting that expression of HSP70 stimulates angiogenesis in granulation tissue. Again, the background number of vessels was similar for the different types of mouse (Fig. 2F and G).

The results in Fig. 2 suggest that expression of HSP70 accelerates gastric ulcer healing through stimulation of cell proliferation at the gastric ulcer margin and angiogenesis in granulation tissue. In order to understand the molecular mechanism, we examined the effect of the expression of HSP70 on the expression of growth factors, which stimulate cell proliferation at the gastric ulcer margin and angiogenesis in granulation tissue. As shown in Fig. 3A, the expression of bFGF, IGF and TGF-β1 mRNAs in the stomach was induced in ulcerated tissues, with the extent of induction being significantly greater in transgenic mice expressing HSP70 compared to wild-type mice. The gastric level of PGE_2 was also elevated in ulcerated tissues and the magnitude of this elevation was significantly greater in transgenic mice expressing HSP70 than in wild-type mice (Fig. 3B). We also found that the level of VEGF in ulcerated tissues was higher in transgenic mice than in wild-type mice (Fig. 3B). These results suggest that the high levels of these growth factors and PGE_2 are responsible for the observed HSP70-dependent acceleration of gastric ulcer healing.

3.3. Effect of GGA on gastric ulcer healing

As described in Section 1, GGA, a clinically used anti-ulcer drug, is an HSP-inducer. Thus, the results described above suggest that administration of GGA could stimulate gastric ulcer healing through the induction of HSP70 expression.

To test this idea, we first focused on HSP70 produced after the development of gastric ulcers, and therefore once daily administration of GGA was started at day 3. As shown in Fig. 4A, the legion index was lower for mice treated with GGA than for non-treated mice at both days 6 and 8, showing that administration of GGA accelerates gastric ulcer healing. Immunoblot analysis confirmed that the expression of HSP70 was induced by the GGA (Fig. 4B and C). Immunohistochemical analysis revealed that a GGA-induced expression of HSP70 took place both at the gastric ulcer margin and

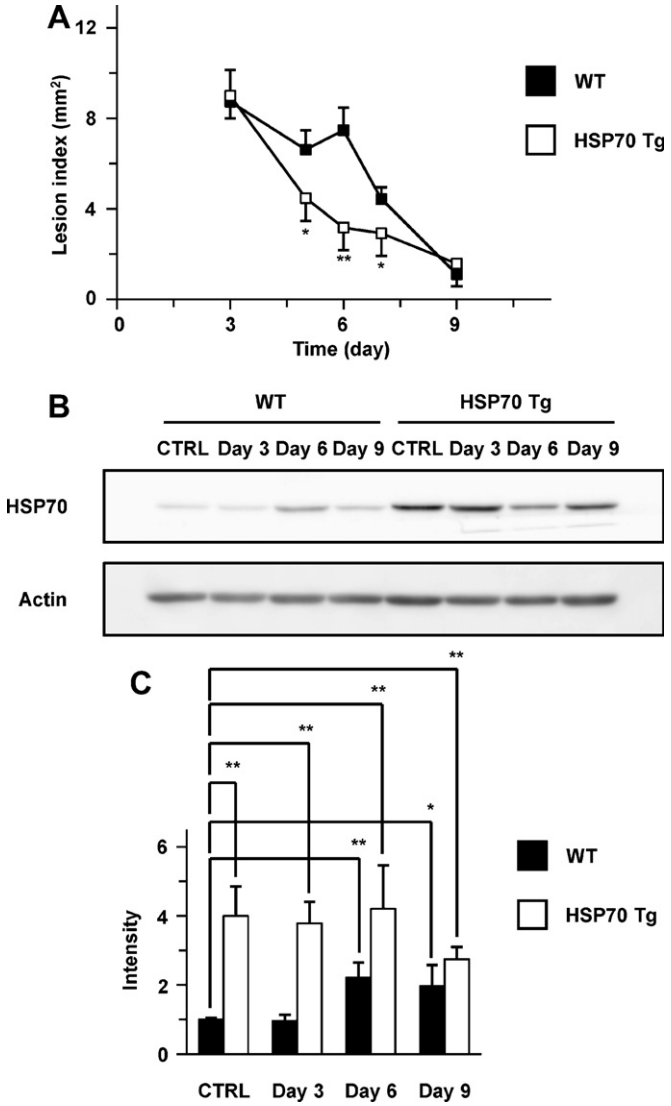

Fig. 2. Effect of expression of HSP70 on gastric ulcer healing. Gastric ulcers were induced in transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) as described in the legend of Fig. 1. (A–G). Hemorrhagic damage (A) and expression of HSP70 (B) were monitored as described in the legend of Fig. 1. The intensity of the HSP70 band was determined, normalized to that of actin and expressed relative to the control sample (C). Sections of gastric tissue were prepared at day 4 (D) or 6 (F) and subjected to immunohistochemical analysis with an antibody against BrdU (D) or CD31 (F). The lower panel in each group is a twice-magnified image of the boxed area in the higher panel (F). The ratio of BrdU-positive cells to total cells (200–400 cells) was determined (E). The number of vessels in a distinct area (0.09 mm^2) was counted (G). Values are mean ± S.E.M. (n = 3–13) $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant. Scale bar, 200 μm.

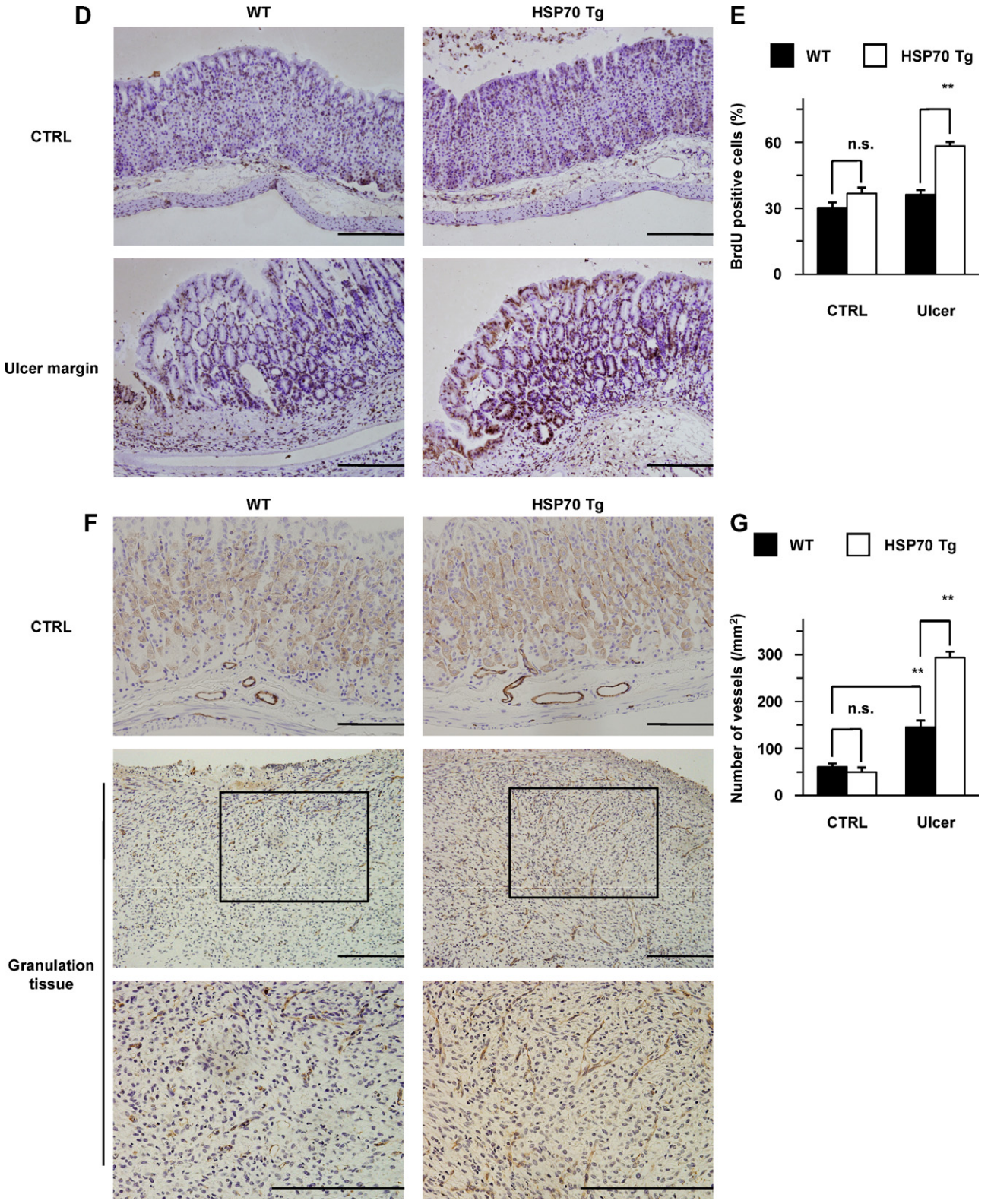

Fig. 2. (*Continued*).

in granulation tissue (Fig. 4D). To test the involvement of HSP70 in this stimulative effect of GGA on gastric ulcer healing, we examined the effect of pre-administration of quercetin (an inhibitor of expression of HSP70). As shown in Fig. 4E, pre-administration of quercetin diminished the stimulative effect of GGA on gastric ulcer healing, suggesting that GGA stimulates gastric ulcer healing through the induction of HSP70 expression. We also examined the effect of oral administration of purified recombinant HSP70 (from days 3 to 6) on gastric ulcer healing. This administration, however, did not affect the process (Fig. 4F).

We then focused our attention on HSP70 produced before the development of gastric ulcers since the background expression of

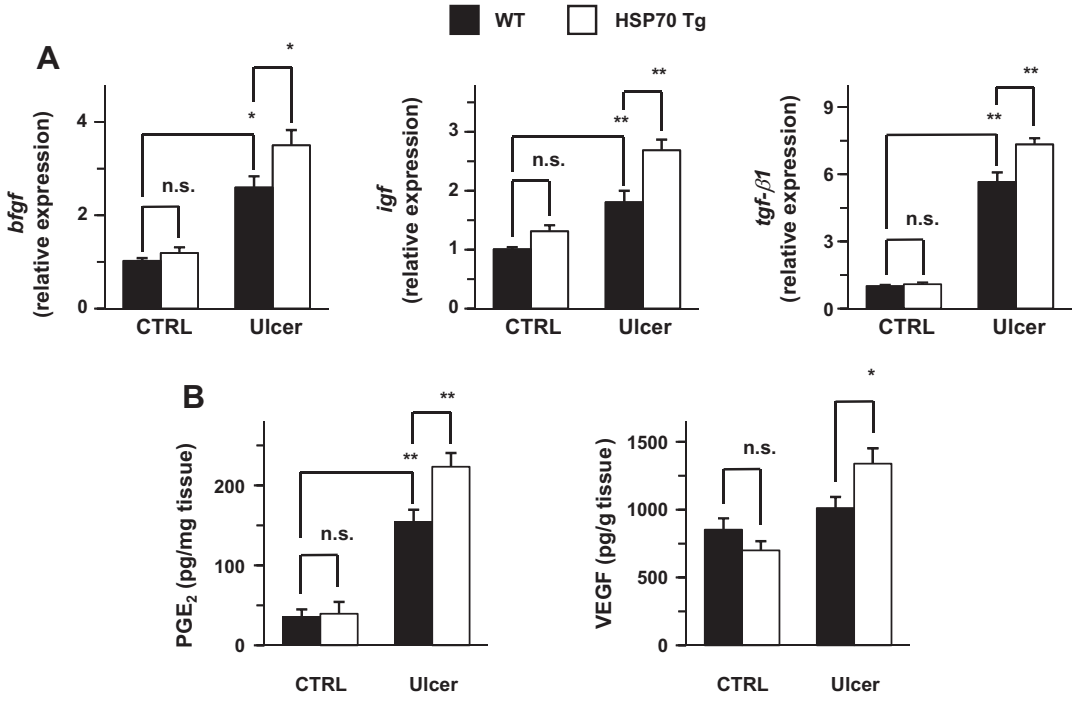

Fig. 3. Effect of expression of HSP70 on factors stimulating gastric ulcer healing. Gastric ulcers were induced in transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) as described in the legend of Fig. 1. (A and B) Total RNA was extracted at day 6 and subjected to real-time RT-PCR using a specific primer set for each gene. Values were normalized to the *gapdh* gene and expressed relative to the control sample (A). The gastric level of PGE_2 at day 3 or VEGF at day 6 was determined by EIA or ELISA, respectively (B). Values are mean ± S.E.M. (n = 6–19) $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant.

HSP70 in the absence of ulcers was also higher in the transgenic mice expressing HSP70 than in the wild-type mice. For this purpose, GGA was administered once only, 2 h before the induction of gastric ulcers. As shown in Fig. 5A, the legion index was lower for mice pre-treated with GGA than for un-treated mice at day 6 but not at day 3, showing that this administration of GGA also accelerated gastric ulcer healing but did not affect the development of gastric ulcers. Immunoblot and immunohistochemical analyses confirmed that GGA induced the expression of HSP70 at the gastric mucosa (Fig. 5B–D). Furthermore, in contrast to the results in Fig. 4F, oral administration of recombinant purified HSP70 (from day 0 to day 3) decreased the legion index at day 6 in a dose-dependent manner, showing that this administration stimulated gastric ulcer healing (Fig. 5E). To address the possibility that contaminated endotoxin but not HSP70 itself was responsible for this stimulation, the HSP70 fraction was denatured by boiling (100 °C for 1 h). It has previously been reported that this treatment diminishes the ability of HSP70, but not of endotoxin, to induce an innate immune response [15,16]. As shown in Fig. 5F, the boiled HSP70 fraction was inert for the stimulation of gastric ulcer healing. The results in Fig. 5 suggest that extracellular HSP70 is able to stimulate gastric ulcer healing.

4. Discussion

Identification of gastric mucosal defensive factors and understanding the molecular mechanisms underlying their actions are important in establishing clinical protocols for the treatment of gastric lesions. PGs, especially PGE_2, have been paid much attention as major defensive factors. HSP70 has also recently been identified as another major defensive factor. For the treatment of gastric ulcers it is important not only to prevent further ulcer formation, but also to enhance ulcer healing. It has been reported that PGE_2 not only prevents the formation of irritant-induced gastric ulcers but also enhances gastric ulcer healing [2,18,20]. As for HSP70, while it has become clear that expression of HSP70 prevents formation of irritant-induced gastric lesions, its role in gastric ulcer healing has been unclear. In this study, we have examined the role of HSP70 in gastric ulcer healing using transgenic mice expressing HSP70 and in response to treatment with GGA.

The expression of HSP70 was found to be induced during gastric ulcer healing. The induction was apparent at days 6 and 9, when ulcer healing progresses, and induction was observed both at the gastric ulcer margin and in granulation tissue, both of which are important regions for ulcer healing. These results suggest that this induction of expression of HSP70 plays an important role in gastric ulcer healing. Similar induction of expression of HSP70 during gastric ulcer healing has been reported elsewhere [31].

We found that gastric ulcer healing is accelerated in transgenic mice expressing HSP70, compared to wild-type mice. Furthermore, both cell proliferation at the gastric ulcer margin and angiogenesis in granulation tissue were accelerated in the transgenic mice. This is the first genetic evidence of a stimulative effect of HSP70 on gastric ulcer healing. Supporting this notion, we found that oral administration of GGA, an inducer of HSPs, stimulates gastric ulcer healing (see below). It was previously reported that pioglitazone, a specific ligand of peroxisome proliferator-activated receptor-gamma, accelerates gastric ulcer healing and induces expression of HSP70 in rats [32]. The results of this study could be extended to suggest that the HSP70 expression induced by pioglitazone is responsible for the acceleration of gastric ulcer healing induced by this drug.

As described in Section 1, increases in the gastric levels of PGE_2 and growth factors (such as bFGF, IGF, TGF-β1 and VEGF) accelerate gastric ulcer healing through enhancement of cell proliferation at the gastric ulcer margin and of angiogenesis in granulation tissue [17]. We have confirmed that expression of these growth factors (except VEGF), and the level of PGE_2, increase during gastric ulcer healing. We also found that these

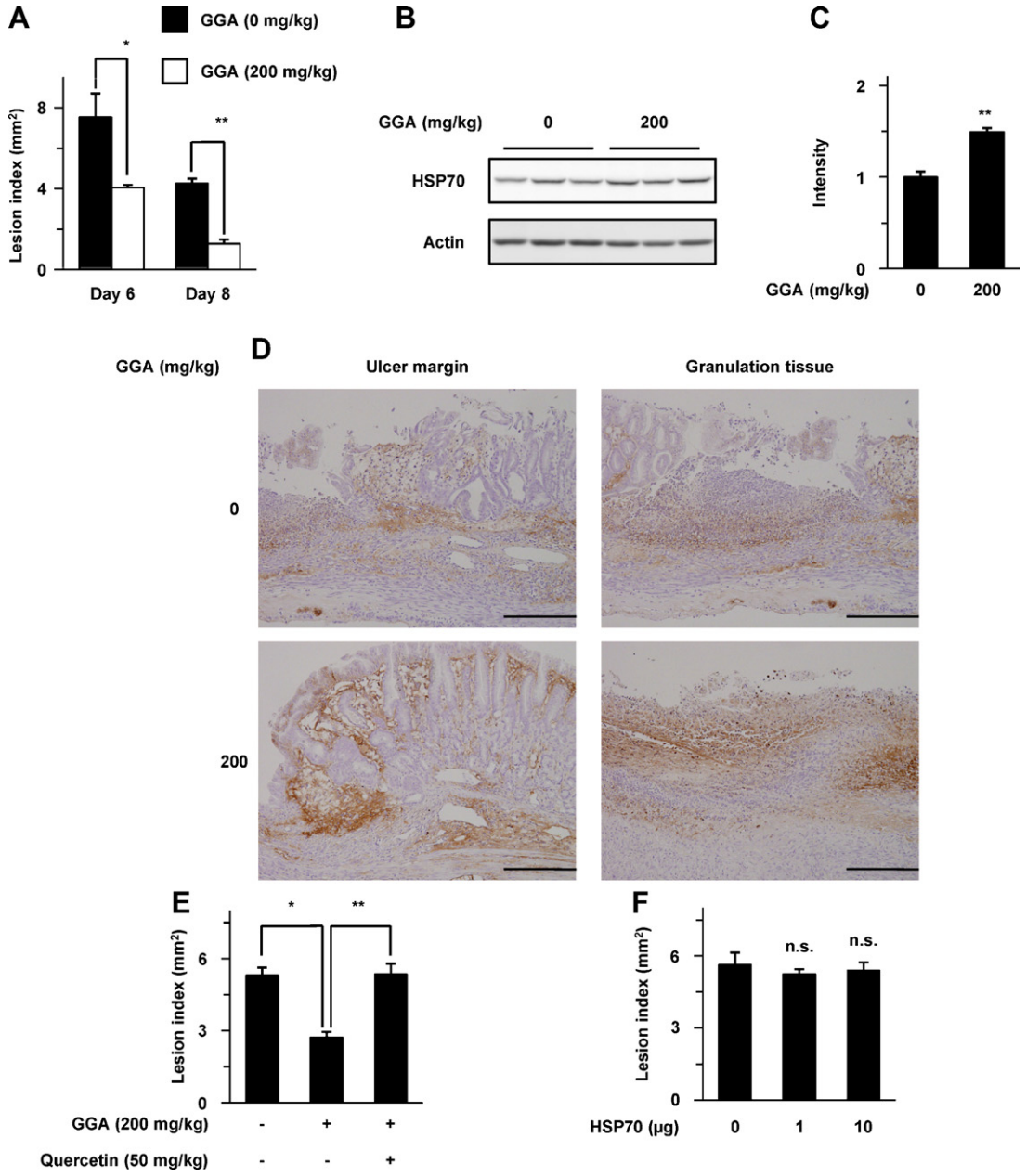

Fig. 4. Effect of GGA on expression of HSP70 and gastric ulcer healing. Gastric ulcers were induced in wild-type mice as described in the legend of Fig. 1. (A–F) Mice were orally administered 200 mg/kg of GGA (10 ml/kg as an emulsion with 5% gum arabic) once daily from day 3 to day 6 or day 8 (A). Mice were orally pre-administered 50 mg/kg of quercetin (10 ml/kg in water) 2 h before each GGA administration (E). Stomachs were removed 2 h after the final administration of GGA or purified HSP70, and hemorrhagic damage (A, E, F) and expression of HSP70 (B–D) monitored as described in the legend of Fig. 1. Values are mean ± S.E.M. (n = 3–7) $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant. Scale bar, 200 μm.

increases were further enhanced in transgenic mice expressing HSP70, suggesting that expression of HSP70 stimulates gastric ulcer healing by increasing the levels of these growth factors and PGE_2.

Both the background (without the development of gastric ulcers) and ulcer-induced expression of HSP70 were higher in transgenic mice expressing HSP70 than in wild-type mice. In order to evaluate the contribution of these HSP70 expression to the stimulation of gastric ulcer healing, we used the HSP70 inducer GGA, and found that its administration either prior to (at day 0) or after (from day 3) the development of gastric ulcers stimulated not only gastric expression of HSP70 but also gastric ulcer healing. These results suggest that both the background and ulcer-induced expression of HSP70 contributes to the stimulation of gastric ulcer healing in transgenic mice expressing HSP70. Furthermore, since HSP70 functions in both intracellular and extracellular compartments, we used the method of oral administration of purified HSP70 to examine the function of extracellular HSP70 in gastric ulcer healing. Administration of the protein from day 0 to day 3, but not from day 3 to day 6, stimulated gastric ulcer healing, suggesting that extracellular HSP70, that is HSP70 released from gastric cells, could enhance gastric ulcer healing at an early rather than a late stage. This idea is supported by the observation that administration of GGA but not purified HSP70 after the development of gastric ulcer stimulated the ulcer healing (Fig. 4A and F), because GGA may increase both intracellular and extracellular HSP70, however, administration of purified HSP may increase the only extracellular one.

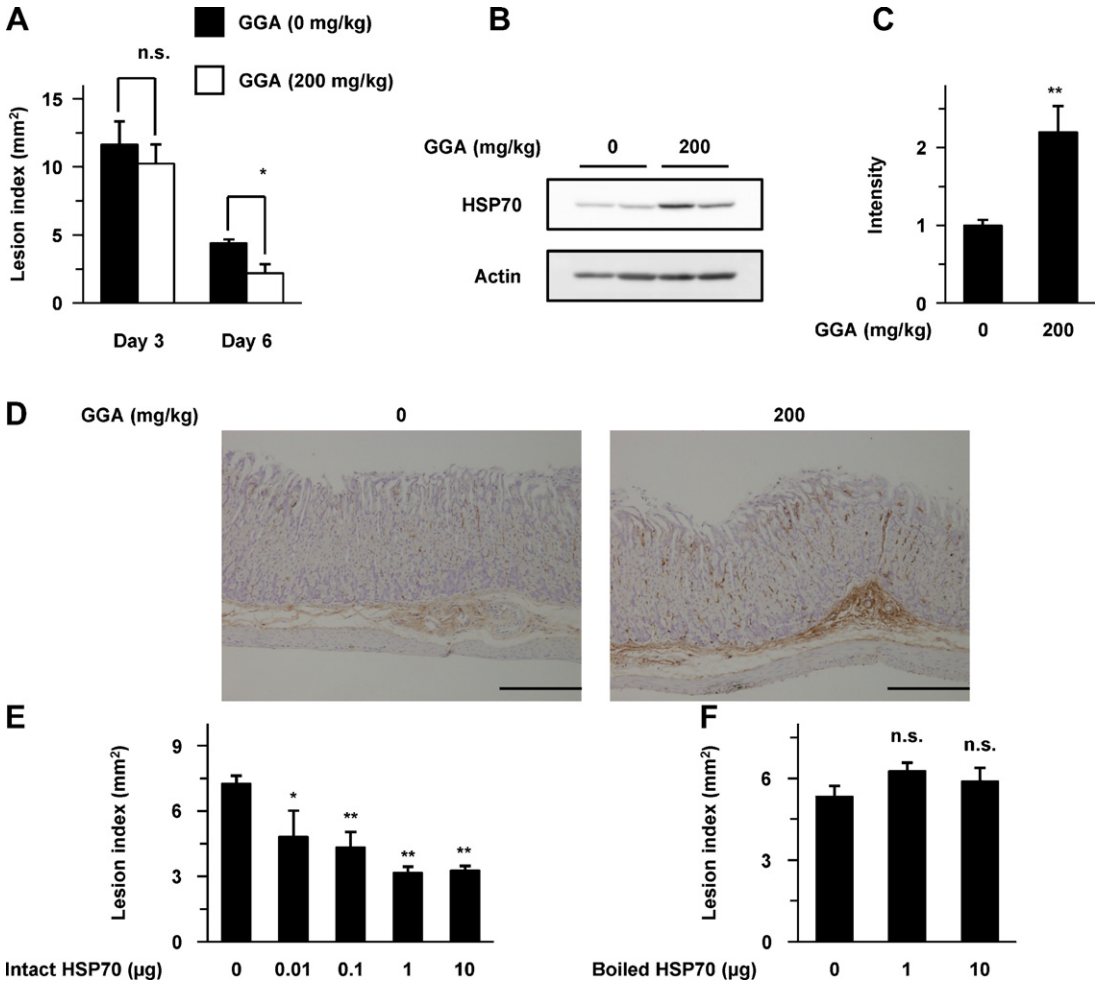

Fig. 5. Effect of GGA and HSP70 protein on gastric ulcer healing. Gastric ulcers were induced in wild-type mice as described in the legend of Fig. 1. (A–F) Mice were orally administered 200 mg/kg of GGA (10 ml/kg as an emulsion with 5% gum arabic) once only at day 0 (2 h before ulcer formation) (A–D). Mice were orally administered the indicated doses of intact purified recombinant HSP70 (E) or boiled HSP70 (F) (100 μl/mouse in PBS) once daily from day 0 to day 3. Stomachs were removed at day 0 (B–D, 2 h after the administration of GGA), day 3 (A) or day 6 (A, E, F), and hemorrhagic damage (A, E, F) and expression of HSP70 (B–D) were monitored as described in the legend of Fig. 1. Values are mean ± S.E.M. (n = 3–9) $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant. Scale bar, 200 μm.

In the early stages of gastric ulcer healing, the inflammatory response, which results in an increase in the level of PGE_2, induces expression of growth factors which stimulate cell proliferation at the gastric ulcer margin and angiogenesis in granulation tissue. Recent studies have revealed that extracellular HSP70 stimulates the innate immune response through its high-affinity receptors, including toll-like receptors, and activates nuclear factor kappa B [13–16]. It has also been reported that necrotic, but not apoptotic, cell death results in the release of intracellular HSPs [11,33]. Furthermore, although toll-like receptors play important roles in innate immunity, recent studies have revealed that their activation stimulates wound healing through various mechanisms including that via an increase in the levels of growth factors (such as VEGF) and the resulting activation of cell migration, proliferation and angiogenesis [34–38]. Thus we propose that HSP70 expressed at background levels (before the formation of a gastric ulcer) is released during the necrotic cell death associated with gastric ulcer formation to activate toll-like receptors, resulting in the stimulation of gastric ulcer healing. This notion is consistent with the idea that HSPs are major components of damage-associated molecular patterns (DAMPs), that are normally hidden in the interior of cells and are released from necrotic cells to stimulate the innate immune system [33].

In the late stages of ulcer healing, in addition to the stimulation of cell proliferation at the gastric ulcer margin and angiogenesis in granulation tissue, it is important to suppress the excessive inflammatory response and resulting cell death. Considering the cytoprotective and anti-inflammatory functions of intracellular HSP70, it is reasonable to speculate that HSP70 produced after the development of gastric ulcers does indeed stimulate gastric ulcer healing by suppressing these processes. The result also suggests that the induction of expression of HSP70 during gastric ulcer healing in wild-type mice contributes to gastric ulcer healing.

GGA was developed in 1984 as an anti-ulcer drug and a number of previous studies have revealed that GGA not only protects the gastric mucosa against irritant-induced lesions but also stimulates the ulcer healing process [39,40]. In addition to various gastroprotective actions, such as increasing gastric mucosal blood flow, stimulation of surface mucus production and direct protection of gastric mucosal cell membranes [41–43], we recently revealed that the HSP-inducing ability of GGA also contributes to the protective effect of GGA against irritant-induced gastric lesions [5,6]. In this study, we have shown that GGA enhances the expression of HSP70 in ulcerated tissues and improves gastric ulcer healing. Taken together with our results obtained with transgenic mice expressing HSP70, we propose that the HSP70-inducing ability of GGA contributes to its stimulative effect on gastric ulcer healing.

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