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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′-gctggac-cagagacccttg-3′, 5′-gctccggaagcaacactca-3′; TGF-β1, 5′-tgacgt-cactggagtacgg-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

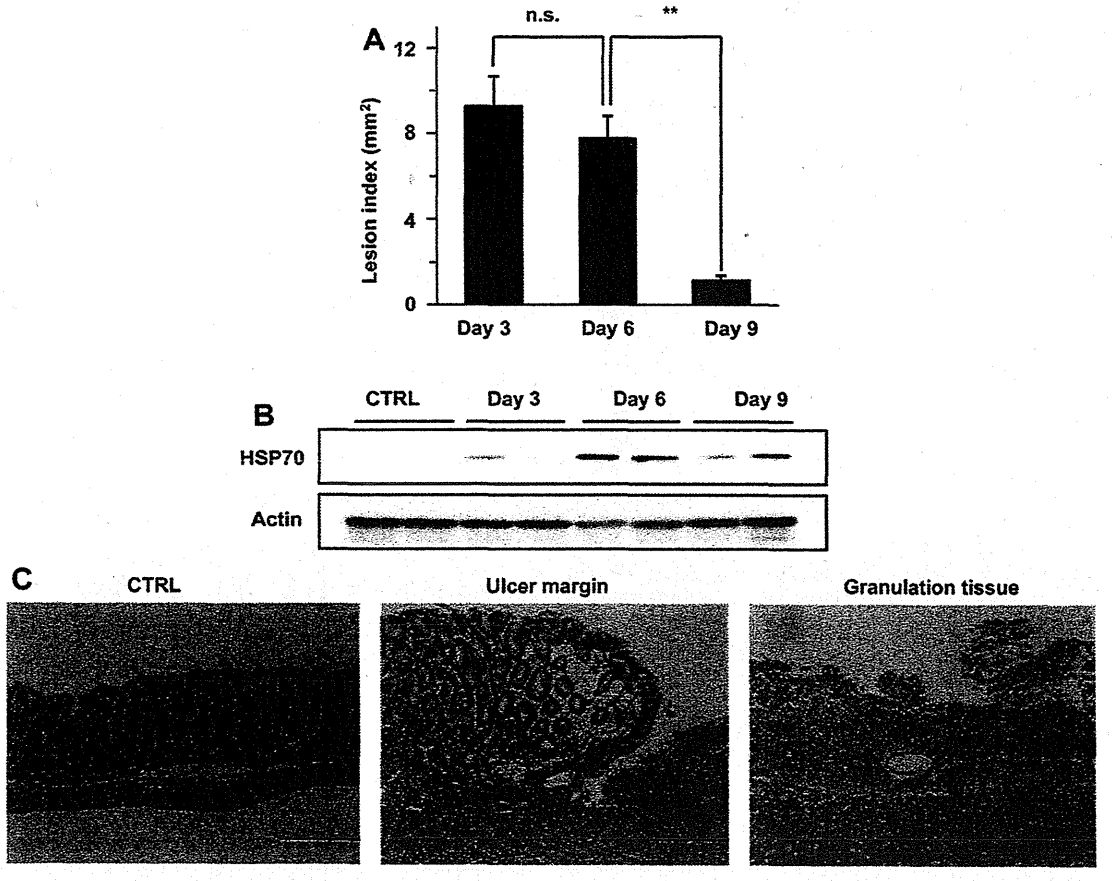

Fig. 1. Expression of HSP70 during gastric ulcer healing. Gastric ulcers were induced in wild-type mice by exposure of the luminal side of their stomachs to acetic acid at day 0, as described in Section 2, and the stomachs were removed at day 3, 6 or 9. Normal stomachs (without ulcer induction) (CTRL) 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

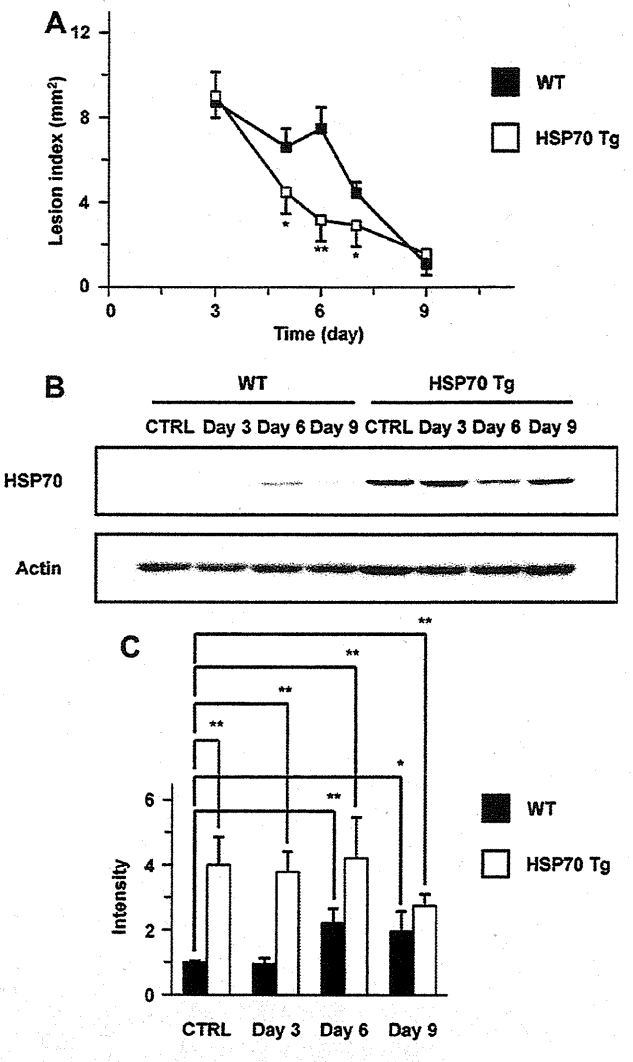

Fig. 2. Effect of expression of HSP70 on gastric ulcer healing. Gastric ulcers were induced in transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) as described in the legend of Fig. 1. (A–G). Hemorrhagic damage (A) and expression of HSP70 (B) were monitored as described in the legend 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.

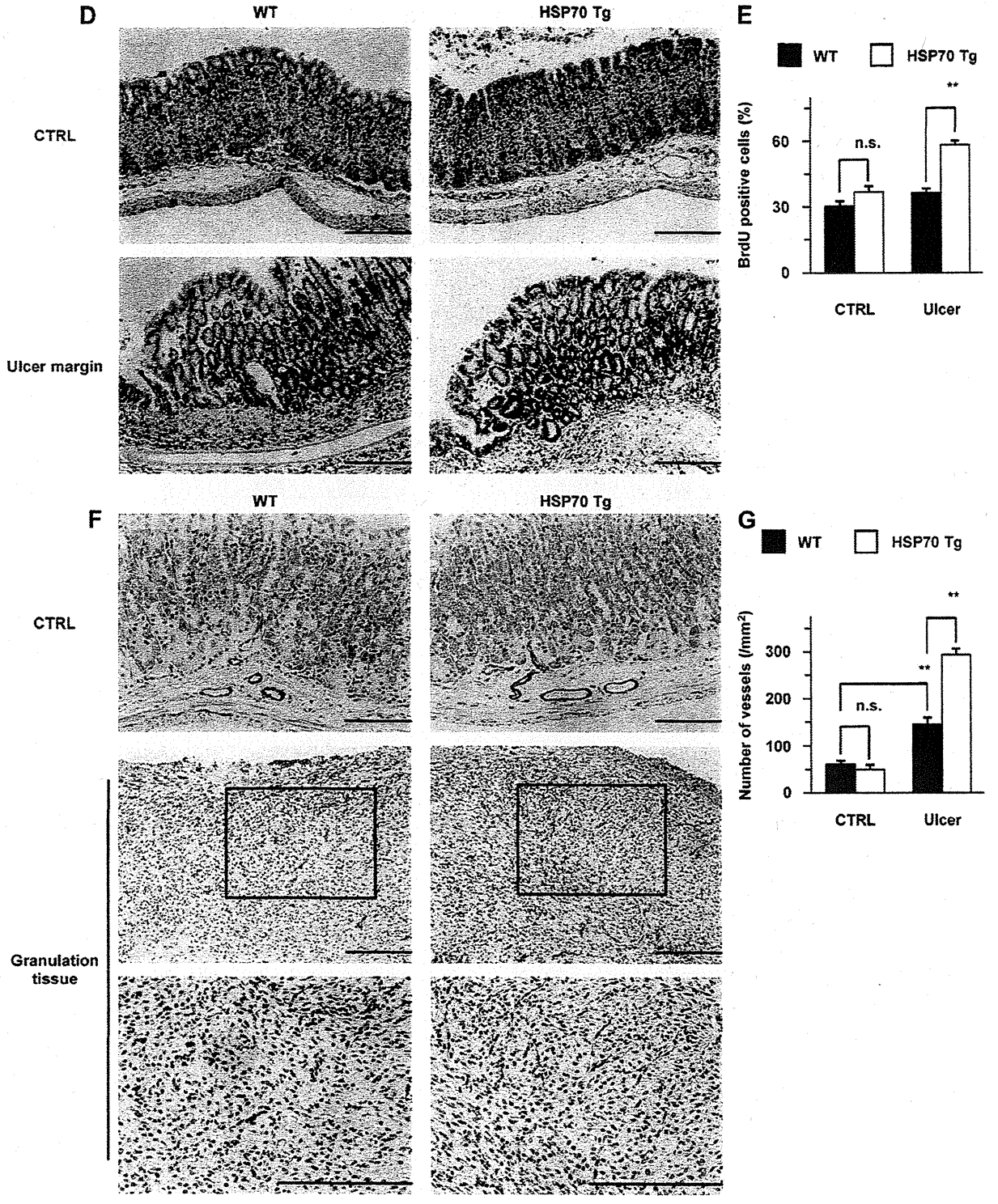

Fig. 2. (*Continued*).

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

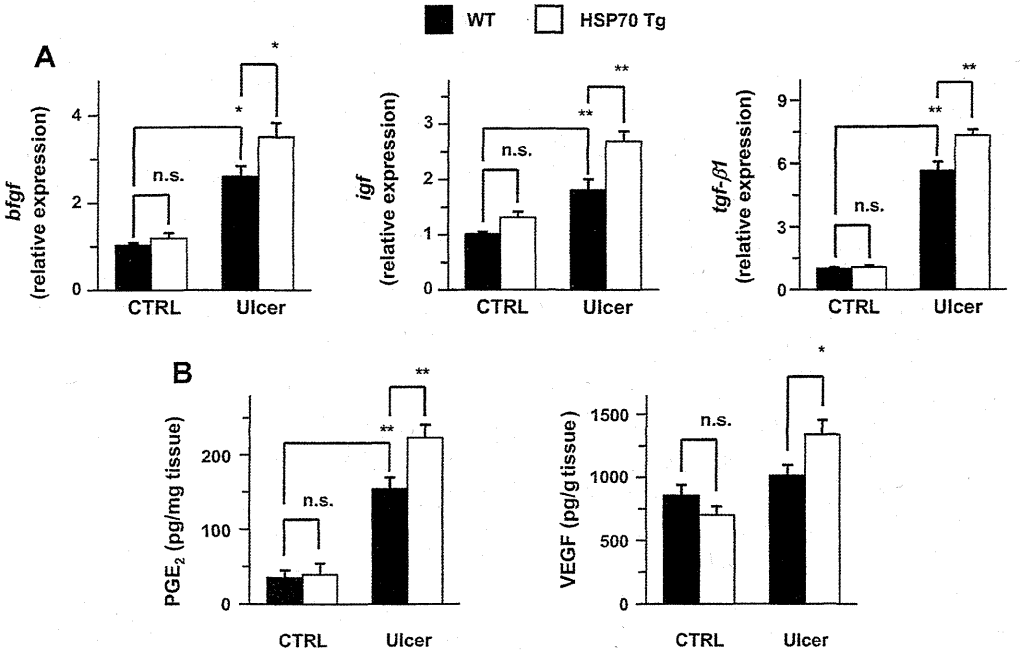

Fig. 3. Effect of expression of HSP70 on factors stimulating gastric ulcer healing. Gastric ulcers were induced in transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) as described in the legend of Fig. 1. (A and B) Total RNA was extracted at day 6 and subjected to real-time RT-PCR using 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

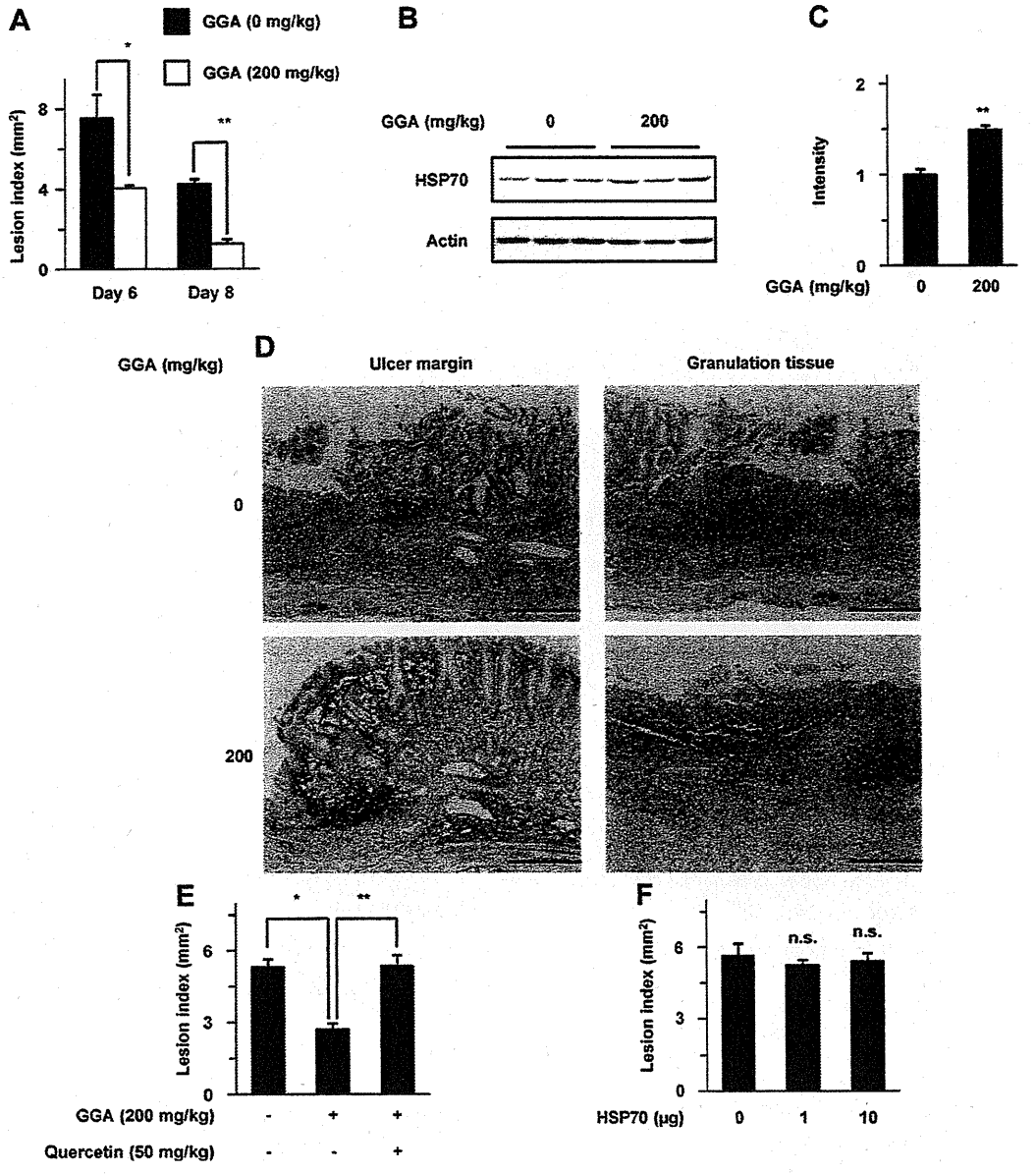

Fig. 4. Effect of GGA on expression of HSP70 and gastric ulcer healing. Gastric ulcers were induced in wild-type mice as described in the legend of Fig. 1. (A–F) Mice were orally administered 200 mg/kg of GGA (10 ml/kg as an emulsion with 5% gum arabic) once daily from day 3 to day 6 or day 8 (A). Mice 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.

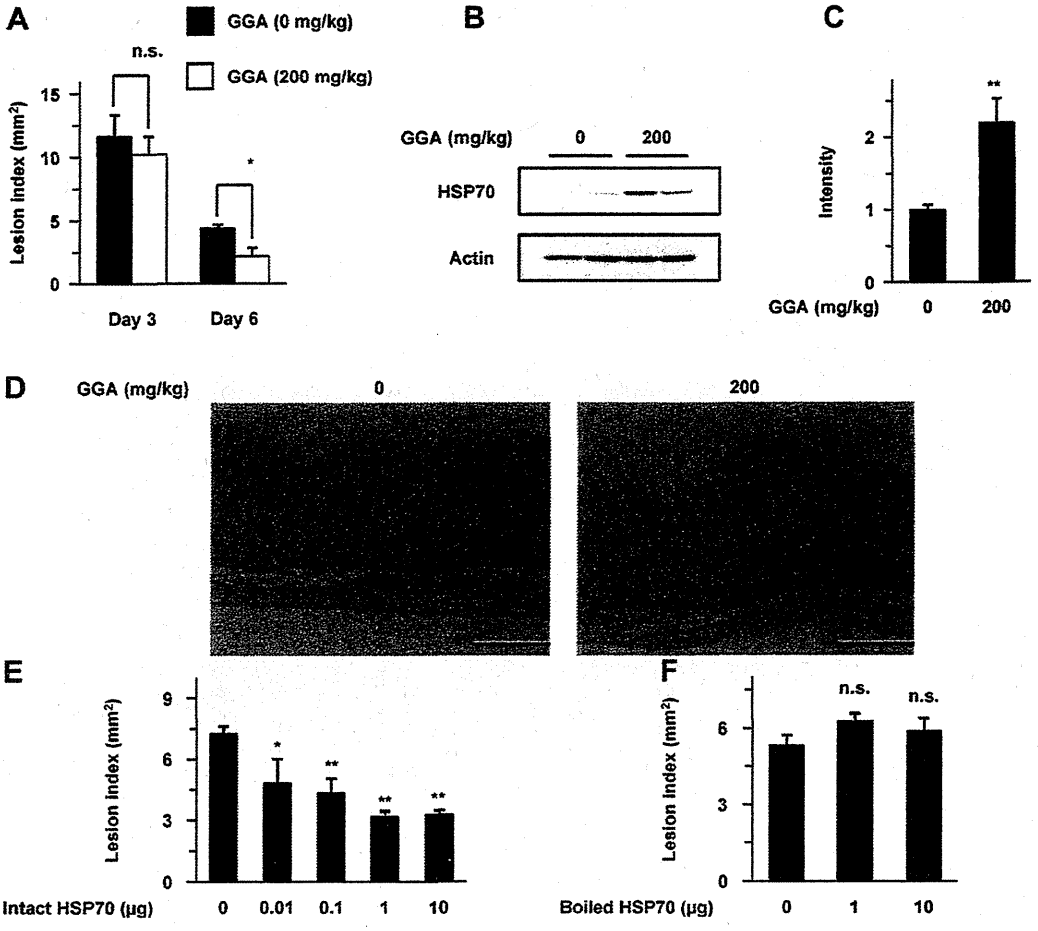

Fig. 5. Effect of GGA and HSP70 protein on gastric ulcer healing. Gastric ulcers were induced in wild-type mice as described in the legend of Fig. 1. (A–F) Mice were orally administered 200 mg/kg of GGA (10 ml/kg as an emulsion with 5% gum arabic) once only at day 0 (2 h before ulcer formation) (A–D). 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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Suppression of Expression of Heat Shock Protein 70 by Gefitinib and Its Contribution to Pulmonary Fibrosis

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Abstract

Drug-induced interstitial lung disease (ILD), particularly pulmonary fibrosis, is of serious clinical concern. Gefitinib, a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR), is beneficial as a drug for treating non-small cell lung cancer; however, this drug induces ILD and the molecular mechanisms underpinning this condition remain unclear. We recently reported that expression of heat shock protein 70 (HSP70) protects against bleomycin-induced pulmonary fibrosis, an animal model of pulmonary fibrosis. In this study, we have examined the effects of drugs known to induce ILD clinically on the expression of HSP70 in cultured lung epithelial cells and have found that gefitinib has a suppressive effect. Results of a luciferase reporter assay, pulse-labelling analysis of protein and experiments using an inhibitor of translation or transcription suggest that gefitinib suppresses the expression of HSP70 at the level of translation. Furthermore, the results of experiments with siRNA for Dicer1, an enzyme responsible for synthesis of microRNA, and real-time RT-PCR analysis suggest that some microRNAs are involved in the gefitinib-induced translational inhibition of HSP70. Mutations in the EGFR affect the concentration of gefitinib required for suppressing the expression of HSP70. These results suggest that gefitinib suppresses the translation of HSP70 through an EGFR- and microRNA-mediated mechanism. *In vivo*, while oral administration of gefitinib suppressed the pulmonary expression of HSP70 and exacerbated bleomycin-induced pulmonary fibrosis in wild-type mice, these effects were not as distinct in transgenic mice expressing HSP70. Furthermore, oral co-administration of geranylgeranylacetone (GGA), an inducer of HSP70, suppressed gefitinib-induced exacerbation of bleomycin-induced pulmonary fibrosis. Taken together, these findings suggest that gefitinib-induced exacerbation of bleomycin-induced pulmonary fibrosis is mediated by suppression of pulmonary expression of HSP70 and that an inducer of HSP70 expression, such as GGA, may be therapeutically beneficial for the treatment of gefitinib-induced pulmonary fibrosis.

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Introduction

Interstitial lung disease (ILD), in particular interstitial pneumonia associated with pulmonary fibrosis, is a devastating chronic lung condition with poor prognosis. Pulmonary fibrosis progresses insidiously, with acute exacerbation of interstitial pneumonia being a highly lethal clinical event [1], [2]. Although most cases of pulmonary fibrosis are idiopathic, some are due to drug side effects (drug-induced pulmonary fibrosis). For example, the anti-tumour drugs gefitinib and imatinib, as well as anti-rheumatoid arthritis drugs such as leflunomide, are known to induce ILD (pulmonary fibrosis). This is cause for serious clinical concern, as it restricts the therapeutic use of these drugs [3], [4], [5]. Unfortunately, the etiology of drug-induced ILD (pulmonary fibrosis) is not yet understood and, as a result, an appropriate animal model has not yet been established. Understanding the mechanism governing drug-induced ILD (pulmonary fibrosis) and developing a viable animal model are therefore important to establish not only a clinical protocol for its treatment but also an assay system that will facilitate screening in order to eliminate candidate drugs with the potential to produce this type of side effect. Bleomycin-induced pulmonary fibrosis in animals mimics some characteristics of human pulmonary fibrosis [6]. We recently reported that leflunomide exacerbates bleomycin-induced pulmonary fibrosis, proposed that this model is a suitable animal model for drug-induced ILD, and suggested that this exacerbation is mediated by epithelial-mesenchymal transition (EMT) of lung epithelial cells [7]. However, the molecular mechanisms underpinning ILD (pulmonary fibrosis) induced by drugs other than leflunomide remain unclear.

Pulmonary fibrosis is induced by repeated epithelial cell damage by reactive oxygen species (ROS) and other stressors and abnormal wound repair and remodelling, resulting in abnormal deposition of extracellular matrix (ECM) proteins, such as collagen. In addition to the increase in transforming growth factor (TGF)-β1 [8], an increase in the level of lung myofibroblasts has been suggested to play an important role in pulmonary fibrosis [9]. It was previously believed that the sole origin of myofibroblasts is

peribronchiolar and perivascular fibroblasts that transdifferentiate into myofibroblasts [10]. However, recently, it was revealed that some of the lung myofibroblasts in pulmonary fibrosis patients originate from lung epithelial cells via EMT [11], [12], [13], [14].

Gefitinib, a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR), is a new molecular target agent for the treatment of patients with advanced non-small cell lung cancer who fail to respond to chemotherapy [15]. Furthermore, recent clinical studies have shown that this drug is particularly effective for patients with EGFR mutations, which causes constitutive activation of EGFR-dependent intracellular signal transduction [16], [17]. Although gefitinib has been recognised as relatively safe based on data from clinical trials, post-marketing surveillance of patients prescribed with gefitinib in Japan has revealed that 6.8% of patients developed interstitial pneumonia and that, of these, 40% of the patients died [4], [18], [19]. The incidence of gefitinib-induced ILD and its mortality rate are higher in Japan than in Western countries [20], [21]. However, the mechanism governing gefitinib-induced ILD (pulmonary fibrosis) and the reason for this ethnic difference is unknown. Furthermore, contradictory results have been reported regarding the effect of gefitinib on bleomycin-induced pulmonary fibrosis in animals (prevention and exacerbation) [22], [23] and the mechanisms governing these phenomena are unknown.

Different stressors induce cells to express heat shock proteins (HSPs) through transcriptional regulation mediated by a transcription factor, heat shock factor 1 (HSF1), and a *cis*-element located in the *hsp* gene promoter, heat shock element (HSE) [24]. HSPs, especially HSP70, expressed in cultured cells protect these cells against a range of stressors, including ROS, by refolding or degrading denatured proteins produced by the stressors (HSPs function as molecular chaperones) [24], [25]. Interestingly, geranylgeranylacetone (GGA), a leading anti-ulcer drug on the Japanese market, has been reported to be a non-toxic HSP-inducer [26], [27]. In addition to the cytoprotective effects of HSP70, its anti-inflammatory effects have been identified recently [28]. We have shown that through the cytoprotective, anti-inflammatory and molecular chaperone activities, both genetic and pharmacologic (by GGA) induction of expression of HSP70 is protective in animal models of various diseases, such as gastric and small intestinal lesions, inflammatory bowel diseases, ultraviolet light-induced skin damage and Alzheimer's disease [29], [30], [31], [32], [33], [34]. Furthermore, we recently reported that bleomycin-induced lung injury, inflammation, fibrosis and dysfunction are suppressed in transgenic mice expressing HSP70 or in GGA-administered wild-type mice. We also suggested that HSP70 plays this protective role through cytoprotective and inflammatory effects and by inhibiting the production of TGF-β1 and TGF-β1-dependent EMT of lung epithelial cells [35].

As a mechanism for the regulation of gene expression, microRNAs (miRNAs) have been paid much attention recently. miRNAs are short non-coding single-stranded RNA species which bind to complementary regions of the 3' untranslated regions (UTRs) of mRNA resulting in repression of translation and/or stimulation of degradation of mRNA. Primary miRNA transcripts are first processed in the nucleus to produce hairpin RNAs (pre-miRNAs), which are then exported into the cytoplasm, where Dicer1 cuts the hairpin to produce miRNAs [36]. Aberrant expression of miRNAs is associated with pathologic conditions, such as cancer, diabetes and fibrosis [36,37].

In this study, we examined the effect on the expression of HSP70 of drugs known to induce ILD clinically in cultured lung epithelial cells, and found that gefitinib suppresses the expression of HSP70. The results suggest that gefitinib regulates expression of HSP70 at the level of translation through an EGFR- and miRNA-mediated mechanism. We also found that oral administration of gefitinib suppresses pulmonary expression of HSP70 and suggested that this suppression is involved in gefitinib-induced exacerbation of bleomycin-induced pulmonary fibrosis. These results suggest that HSP70 plays an important role in gefitinib-induced ILD (pulmonary fibrosis) and that examination of the effect of drugs on HSP70-expression *in vitro* is useful as a screening system in order to eliminate candidate drugs with the potential to induce ILD.

Materials and Methods

Ethics Statement

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 Institutes of Health, and were approved by the Animal Care Committee of Kumamoto University. Permit numbers or approval ID for this study is C-20–166-R1.

Chemicals and animals

Paraformaldehyde, fetal bovine serum (FBS), 4-(dimethylamino)-benzaldehyde (DMBA), chloramine T, cycloheximide, SP600125, Orange G, RPMI1640 and DMEM were obtained from Sigma (St. Louis, MO). Bleomycin was purchased from Nippon Kayaku (Tokyo, Japan). An RNeasy kit, miScript miRNA Mimic and HiPerFect were obtained from Qiagen (Valencia, CA), the PrimeScript® 1st strand cDNA synthesis kit was from TAKARA Bio (Ohtsu, Japan), and the iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). The mirVana miRNA isolation kit and pMIR-REPORT System were purchased from Applied Biosystems (Carlsbad, CA). Antibodies against actin and HSP70 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and BD Bioscience (San Francisco, CA), respectively. Antibodies against HSP27, HSP47, HSP60 and HSP90 were from Stressgene (San Francisco, CA). The NCode VILO miRNA cDNA synthesis kit and Lipofectamine (TM2000) were obtained from Invitrogen (Carlsbad, CA). A771726, gefitinib, imatinib, amiodarone, L-hydroxyproline, azophloxin and aniline blue were from WAKO Pure Chemicals (Tokyo, Japan). Xylidine ponceau was from WALDECK GmbH & Co. (Muenster, Germany), and Mayer's hematoxylin, 1% eosin alcohol solution, mounting medium for histological examination (malinol) and Weigert's iron hematoxylin were from MUTO Pure Chemicals (Tokyo, Japan). Transgenic mice expressing HSP70 were gifts from Drs. CE Angelidis and GN Pagoulatos (University of Ioannina, Ioannina, Greece) and were crossed with C57BL/6J wild-type mice 10 times to generate the mice used in this study [32].

Cell culture

A549 and H1975 cells, and PC9 cells were cultured in DMEM and RPMI1640 medium, respectively, 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.

Real-time RT-PCR analysis

Real-time RT-PCR was performed as previously described [38] with some modifications. Total RNA was extracted from cells using an RNeasy kit or mirVana miRNA isolation kit according to the manufacturer's protocol. Samples were reverse-transcribed using a first-strand cDNA synthesis kit or NCode VILO miRNA cDNA synthesis kit. Synthesized cDNA was used in real-time RT-PCR experiments (Chromo 4 instrument; Bio-Rad Laboratories) using iQ SYBR GREEN Supermix, and analyzed with Opticon

Monitor Software. Specificity was confirmed by electrophoretic analysis of the reaction products and by the inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, *actin* or RUN44 cDNA was used as an internal standard. Primers were designed using the Primer3 website or NCode™ miRNA database website.

The primers used were (name: forward primer, reverse primer): *hsp27*: 5'-ccacccaagtttcctcctc-3', 5'-gactgggatggtgatctcgt-3'; *hsp47*: 5'-ccatgttcttcaagccacact-3', 5'-cgtagtagttgtagaggcctgt-3'; *hsp60*: 5'-tttcagatggagtggctgtg-3', 5'-caatgccttcttcaacagca-3'; *hsp70*: 5'-aggccaacaagatcaccatc-3', 5'-tcgtcctccgctttgtactt-3'; *hsp90*: 5'-ggcagaggctgataagaacg-3', 5'-ctggggatcttccagactga-3'; *actin*: 5'-ggacttcgagcaagagatgg-3', 5'- agcactgtgttggcgtacag-3'; miR-146a: 5'-tgagaactgaattccatgggtt-3'; miR-146b-5p: 5'-gtgagaactgaattccataggct-3'; miR-223*: 5'-gcgtgtatttgacaagctgagtt-3'; miR-561: 5'-cgcaaagtttaagatccttgaagt-3'; miR-449a: 5'-tggcagtgtattgttagctggt-3'; miR-449b: 5'-aggcagtgtattgttagctggc-3'; RUN44: 5'-gagctaattaagaccttcatgttca-3', 5'-cctggatgatgataagcaaatg-3'. For miRNAs, the universal primer in the NCode VILO miRNA cDNA synthesis kit was used as the reverse primer.

We searched for miRNAs that potentially bind to the 3' UTR of *hsp70*, using the TargetScan and Segal Lab websites.

Luciferase assay

DNA fragments of the *hsp70* 3' UTR (from 2169 to 2427) were amplified by PCR and ligated into the Spe*I*-Hin*dIII* site of the *Photinus pyralis* luciferase reporter plasmid (pMIR-REPORT) to generate pMIR/luc/*hsp70* 3' UTR. The pGL-3/HSE plasmid was constructed by inserting HSE just upstream of the *luciferase* gene. The pGL-3/*hsp70*pro plasmid, which was constructed by inserting the *hsp70* promoter into the same region, was generously provided by Dr. Chang EB (University of Chicago). The luciferase assay was performed as described previously [38]. Transfections were carried out using Lipofectamine (TM2000) according to the manufacturer's instructions. Cells were used for experiments after a 24 h recovery period. Transfection efficiency was determined in parallel plates by transfection of cells with a pEGFP-N1 control vector. Cells were transfected with 0.5 μg of one of the *Photinus pyralis* luciferase reporter plasmids (pMIR/luc/*hsp70* 3' UTR, pGL-3/*hsp70*pro or pGL-3/HSE) and 0.125 μg of the internal standard plasmid bearing the *Renilla reniformis* luciferase reporter (pRL-SV40). *Photinus pyralis* luciferase activity in the cell extracts was measured using the Dual Luciferase Assay System and then normalized for *Renilla reniformis* luciferase activity.

Immunoblotting analysis

Whole cell extracts were prepared as described previously [38]. The protein concentration of the samples was determined by the Bradford method [39]. Samples were applied to polyacrylamide SDS gels, subjected to electrophoresis, and the resultant proteins immunoblotted with their respective antibodies.

Pulse-chase and pulse-labelling analyses

Pulse-chase and pulse-labelling analyses were carried out as described previously [40], with some modifications. Cells were labelled with [^{35}S]methionine and [^{35}S]cysteine in methionine- and cysteine-free RPMI1640 medium for 15 min. To chase labelled proteins, cells were washed with fresh complete (with methionine and cysteine) medium three times and incubated in complete medium for 4 or 8 h. HSP70 was immunoprecipitated with its antibody and separated by SDS-polyacrylamide gel electrophoresis, and visualised by autoradiography (Fuji BAS 2500 imaging analyzer).

Transfection of cells with siRNA or miRNA mimic RNA fragments

The siRNA for Dicer1 and the miRNA mimic RNA fragments for miR-146a and miR-146b-5p were purchased from Qiagen. A549 cells were transfected with these RNAs using HiPerFect transfection reagents according to the manufacturer's instructions. The siRNA (5'-uucuccgaacgugucacgudTdT-3' and 5'-acgugacacguucggagaadTdT-3') was used as a non-specific siRNA.

Administration of bleomycin, gefitinib and GGA

C57BL/6 mice were maintained under chloral hydrate anesthesia (500 mg/kg) and given one intratracheal injection of bleomycin (1 or 2 mg/kg) to induce fibrosis. Gefitinib (200 mg/kg) was dissolved in 1% methylcellulose and administered orally. GGA (200 mg/kg) was dissolved in 5% arabic gum and 0.06% Tween and administered orally.

Histological analysis

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

For histological examination, sections were stained first with Mayer's haematoxylin and then with 1% eosin alcohol solution. Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

For Masson's trichrome staining of collagen, sections were sequentially treated with solution A (5% (w/v) potassium dichromate and 5% (w/v) trichloroacetic acid), Weigert's iron hematoxylin, solution B (1.25% (w/v) phosphotungstic acid and 1.25% (w/v) phosphomolybdic acid), 0.75% (w/v) Orange G solution, solution C (0.12% (w/v) xylidine ponceau, 0.04% (w/v) acid fuchsin and 0.02% (w/v) azophloxin), 2.5% (w/v) phosphotungstic acid, and finally aniline blue solution. Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

Hydroxyproline determination

Hydroxyproline content was determined as previously described [41]. Briefly, the right lung was removed and homogenized in 0.5 ml of 5% TCA. After centrifugation, pellets were hydrolyzed in 0.5 ml of 10 N HCl for 16 h at 110°C. Each sample was incubated for 20 min at room temperature after the addition of 0.5 ml of 1.4% (w/v) chloramine T solution and then incubated at 65°C for 10 min after the addition of 0.5 ml of Ehrlich's reagent (1M DMBA, 70% (v/v) isopropanol and 30% (v/v) perchloric acid). Absorbance was measured at 550 nm and the amount of hydroxyproline was determined.

Statistical analysis

Two-way analysis of variance (ANOVA), followed by the Tukey test or the 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 $P<0.05$.

Results

Suppression of expression of HSP70 by gefitinib

We first examined the effects of drugs known to induce ILD clinically (A771726 (an active metabolite of leflunomide), amiodarone, gefitinib and imatinib) on the expression of HSP70 in cultured human type II alveolar (A549) cells. As shown in Fig. S1, a decrease in the level of HSP70 was observed in cells treated with gefitinib but not in cells treated with other drugs.

As shown in Fig. 1A, treatment of cells with gefitinib decreased the level of not only HSP70 but also HSP90 in a dose-dependent manner; however, the decrease was only clearly observed for HSP70 and not for HSP90, at the concentration of 1 μM. In contrast, levels of other HSPs (HSP27, HSP47 and HSP60) were not affected by treatment with gefitinib (Fig. 1A). Erlotinib is another inhibitor of EGFR used clinically [42] and here we found that this drug also decreases the levels of HSP70 and HSP90 (Fig. 1B), suggesting that the inhibitory effect of gefitinib on EGFR is involved in the decrease in the level of HSP.

Real-time RT-PCR analysis revealed that treatment of cells with gefitinib at 1 μM, a concentration determined to be sufficient to decrease the level of HSP70 (Fig. 1A), did not affect the *hsp70* mRNA level; however, this drug decreased the level at concentrations higher than 10 μM (Fig. 1C).

Suppression of translation of HSP70 by gefitinib

The cellular level of protein is regulated by various factors, such as transcription, mRNA stability, translation and protein stability. With these possibilities in mind, we then examined which of these factors is affected by gefitinib in relation to HSP70. The results in Fig. 1C suggest that 1 μM gefitinib does not affect the transcription and mRNA stability of *hsp70*.

To confirm this, a luciferase reporter assay was used to examine the effect of gefitinib on the promoter activity of *hsp70*. Treatment of cells with gefitinib did not affect the luciferase activity in cells carrying a reporter plasmid with an *hsp70* promoter or an HSE inserted upstream of the *luciferase* gene (Fig. 2A), suggesting that gefitinib does not affect the promoter activity of *hsp70*. Further to this, 1 μM gefitinib did not affect the *hsp70* mRNA level in cells pre-treated with actinomycin D, an inhibitor of transcription (Fig. 2B), suggesting that treatment of cells with 1 μM gefitinib does not affect *hsp70* mRNA stability. The results in Fig. 1A and B also suggest that treatment of cells with 10 μM gefitinib affects *hsp70* mRNA stability but not promoter activity.

We then tested whether gefitinib affects the translation and degradation of HSP70. As shown in Fig. 2C, the decrease in the level of HSP70 with 1 μM gefitinib was not observed in cells pre-

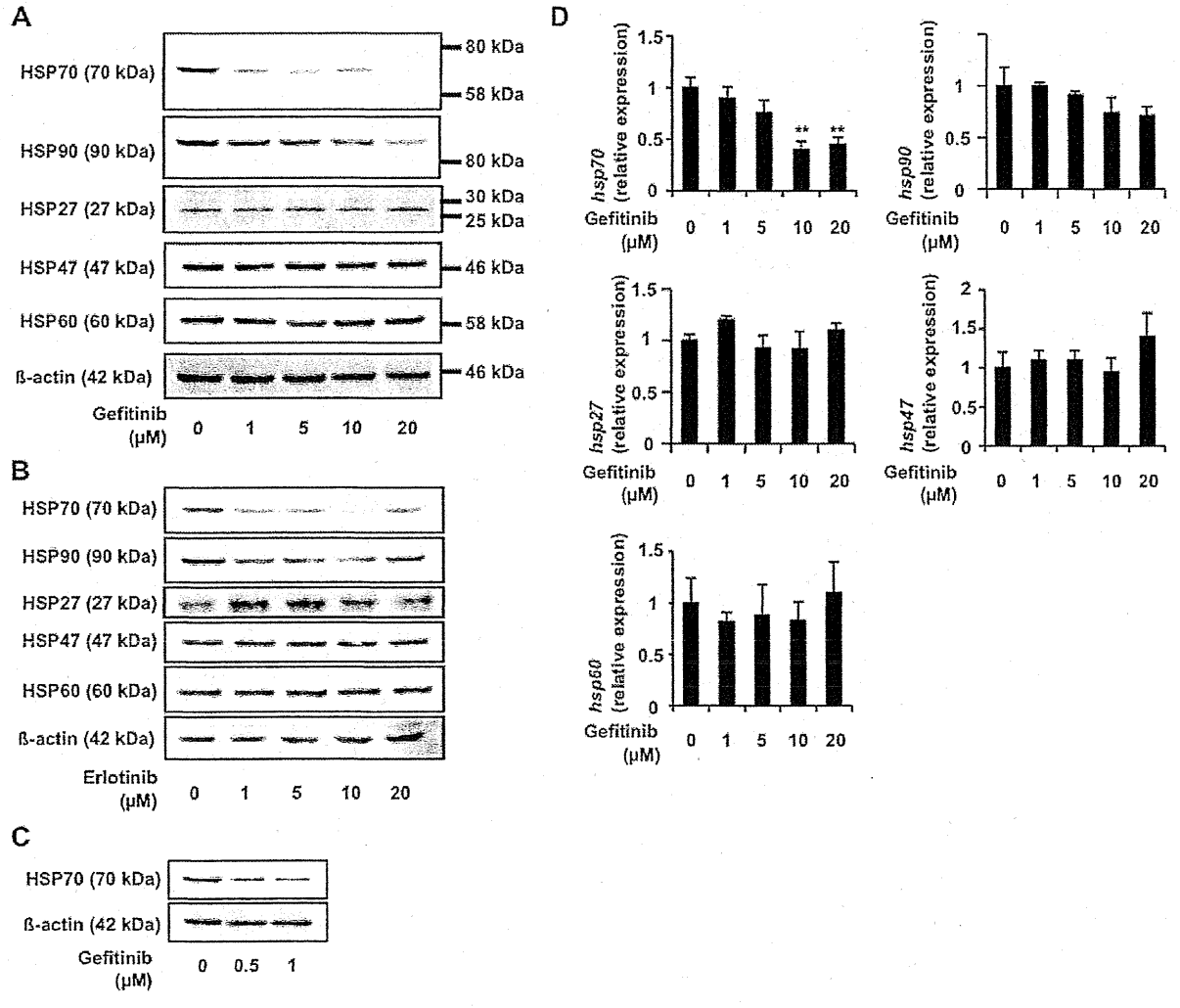


Figure 1. Suppression of expression of HSPs by gefitinib. A549 cells were incubated with the indicated concentration of gefitinib (A, C) or erlotinib (B) for 24 h (A, B) or 12 h (C). Whole-cell extracts were analyzed by immunoblotting with an antibody against HSP70, HSP90, HSP27, HSP47, HSP60 or actin (A, B). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer set for each gene. Values were normalized to the *actin* gene, expressed relative to the control sample (C). Values shown are mean ± S.D. ($n=3$). $^{**}P<0.01$.
doi:10.1371/journal.pone.0027296.g001

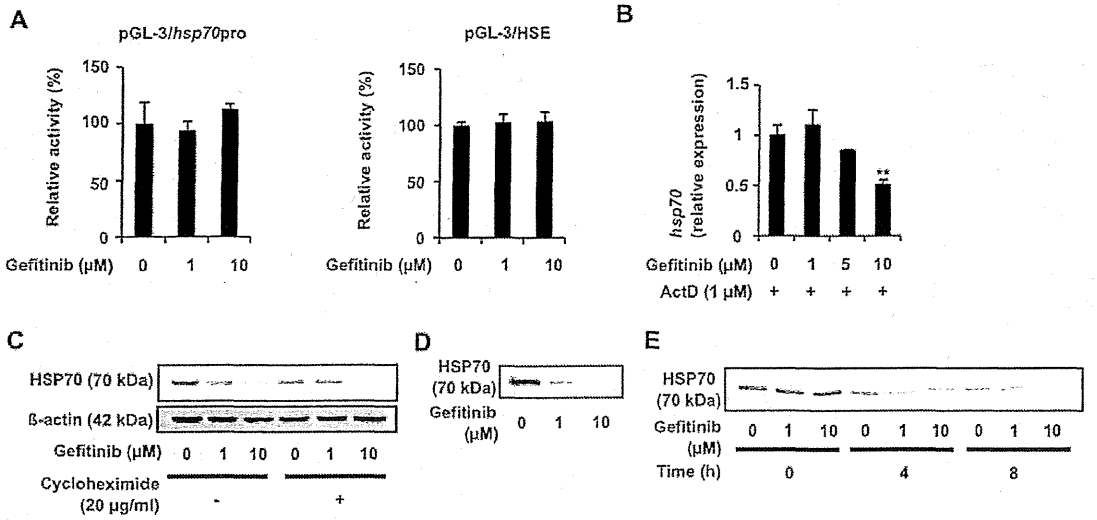


Figure 2. Translational regulation of expression of HSP70 by gefitinib. A549 cells were co-transfected with pRL-SV40 (internal control plasmid carrying the *R. reniformis luciferase* gene) and a pGL-3 derivative (pGL-3/*hsp70*pro or pGL-3/HSE) and cultured for 24 h. Cells were incubated with the indicated concentration of gefitinib for 24 h and *P. pyralis* luciferase activity was measured and normalized for *R. reniformis* luciferase activity. The 100% value of the *P. pyralis* luciferase activity is 6.9×10^4 or 5.8×10^4 units for pGL-3/*hsp70*pro or pGL-3/HSE, respectively (A). A549 cells were pre-incubated with 1 μg/ml actinomycin D (ActD) (B) or 20 μg/ml cycloheximide (C) for 1 h and further incubated for 8 h (B) or 24 h (C) with the indicated concentration of gefitinib (B, C). The mRNA (B) and protein (C) expression was monitored and is expressed as described in the legend of Fig. 1 A549 cells were pulse-labelled for 15 min with [^{35}S]methionine and [^{35}S]cysteine (D, E). Before the pulse-labelling, cells were incubated with the indicated concentration of gefitinib for 8 h (D). Pulse-labelled proteins were chased with excess amounts of Non-radioactively labeled methionine and cysteine for the indicated period in the presence of the indicated concentration of gefitinib (E). Labelled proteins were extracted, immunoprecipitated with antibody against HSP70, subjected to SDS-PAGE and autoradiographed (D, E). Values are mean ± S.D. ($n=3$). **$P<0.01$.
doi:10.1371/journal.pone.0027296.g002

treated with cycloheximide, an inhibitor of protein synthesis. Furthermore, a protein pulse-labelling experiment showed that the synthesis of HSP70 was inhibited in cells pre-treated with gefitinib (Fig. 2D). These results suggest that 1 μM gefitinib inhibits the translation of HSP70. On the other hand, the results of the pulse-chase experiment suggest that treatment of cells with 1 μM gefitinib does not affect the stability of HSP70; 1 μM gefitinib did not affect the level of labelled HSP70 after chase periods of 4 h or 8 h (Fig. 2E). The results in Fig. 2E also suggest that treatment of cells with 10 μM gefitinib stimulates the degradation of HSP70.

3' UTRs of genes play an important role in translational regulation, including that by miRNAs. Thus, we examined the contribution of this region to gefitinib-induced suppression of expression of HSP70 by a luciferase reporter assay. Treatment of cells with gefitinib significantly decreased the luciferase activity in cells carrying the reporter plasmid in which the 3' UTR of *hsp70* was inserted downstream of the *luciferase* gene (Fig. 3A), suggesting that 3' UTR-mediated modulation of HSP70 translation plays an important role in the gefitinib-induced suppression of HSP70 expression.

The contribution of miRNAs to gefitinib-induced suppression of HSP70 expression was then tested by use of siRNA for Dicer1, a protein essential for maturation of miRNAs. As shown in Fig. 3B, transfection of cells with siRNA for Dicer1 decreased the level of *Dicer1* mRNA, but not that of *hsp70*, suggesting that the miRNA system is not involved in the regulation of *hsp70* mRNA stability. The decrease in the level of HSP70 and inhibition of HSP70 protein synthesis in the presence of 1 μM gefitinib were not observed in cells transfected with siRNA for Dicer1 (Fig. 3C and D), suggesting that the 1 μM gefitinib-dependent inhibition of HSP70 translation is mediated by the miRNA system. However, the 10 μM gefitinib-dependent decrease in the level of HSP70 and inhibition of protein synthesis of HSP70 were observed even in cells transfected with siRNA for Dicer1 (Fig. 3C and D).

Next, using a database, we searched for miRNAs with sequences complementary to the 3' UTR of *hsp70*. Six candidate miRNAs were found (miR-146a, miR-146b-5b, miR-223*, miR-561, miR-449a and miR-449b). Real-time RT-PCR analysis revealed that among these miRNAs, the levels of miR-146a and miR-146b-5b clearly increased after treatment of cells with 1 μM gefitinib (we used experimental conditions of real-time RT-PCR under which only fully processed miRNA could be detected) (Fig. 4A). Furthermore, transfection of cells with siRNA for Dicer1 suppressed this gefitinib-dependent increase in the levels of these miRNAs (miR-146a, miR-146b-5b) (Fig. 4B) and transfection of cells with synthesized miR-146a and miR-146b-5b mimic RNA fragments decreased the level of HSP70 (Fig. 4C and D) but not that of *hsp70* mRNA (Fig. 4E). These results suggest that a gefitinib-dependent increase in the levels of miR-146a and miR-146b-5b is involved in gefitinib-dependent inhibition of HSP70 translation.

Involvement of EGFR and JNK inhibition in gefitinib-dependent suppression of expression of HSP70

As described above, gefitinib inhibits self-phosphorylation of tyrosine residues in the cytosolic domains of EGFR. Thus, here we tested whether or not gefitinib suppresses expression of HSP70 through inhibition of the EGFR. To do this we used the cell lines H1975 and PC9 which are resistant or sensitive, respectively, to gefitinib-induced inhibition of the self-phosphorylation of EGFR and the resulting modulation of intracellular signal transduction due to mutations in the EGFR [43], [44]. As shown in Fig. 5A, the concentration of gefitinib required for suppression of HSP70 expression was higher and lower in H1975 cells and PC9 cells,

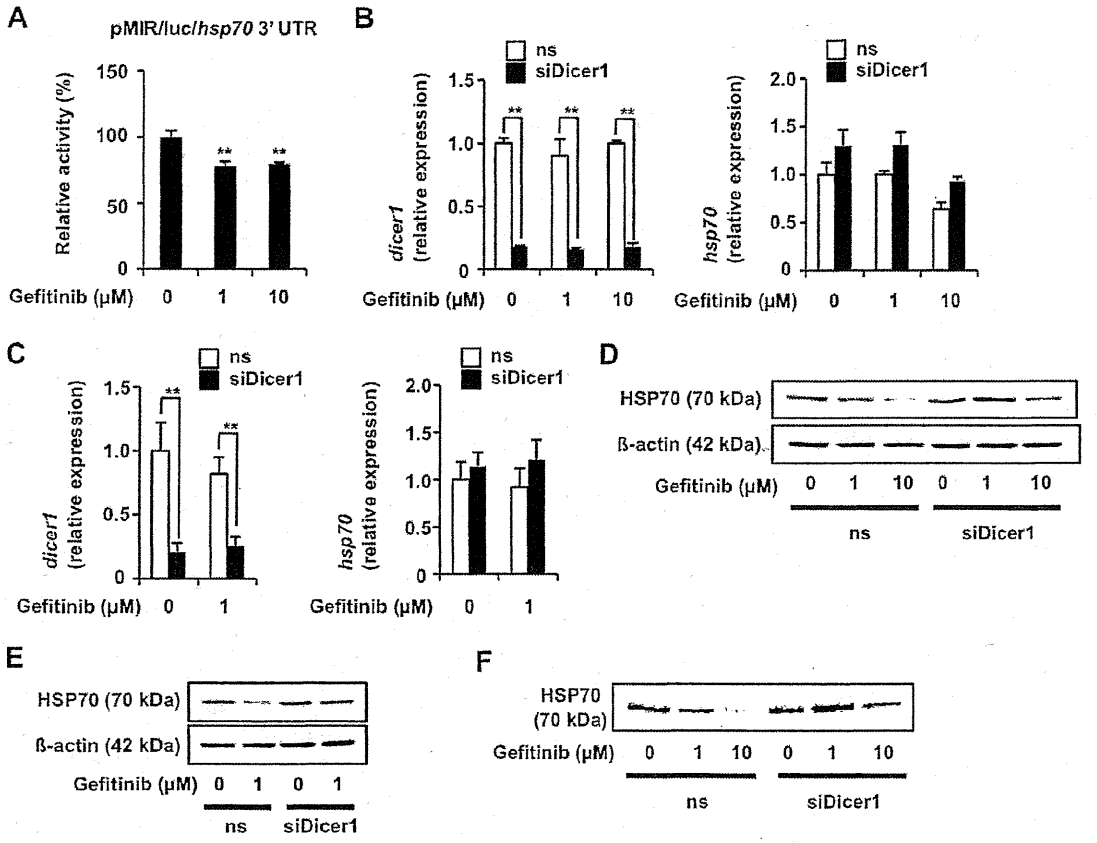


Figure 3. Contribution of miRNA to gefitinib-dependent suppression of expression of HSP70. A549 cells were co-transfected with pRL-SV40 and pMIR/luc/*hsp70* 3′ UTR and cultured for 24 h. Cells were incubated with the indicated concentration of gefitinib for 24 h and luciferase reporter assay was done as described in the legend of Fig. 2. The 100% value of the *P. pyralis* luciferase activity is 1.2×10^5 units (A). A549 cells were transfected with siRNA for Dicer1 (siDicer1) or non-specific siRNA (ns) (B-D). After 24 h, cells were incubated with the indicated concentration of gefitinib for 12 h (B), 24 h (C) or 8 h (D). The mRNA (B) and protein (C) expression was monitored and is expressed as described in the legend of Fig. 1. Pulse-labelling experiments were performed as described in the legend of Fig. 2 (D). Values shown are mean ± S.D. ($n=3$). $^{**}P<0.01$; n.s., not significant.
doi:10.1371/journal.pone.0027296.g003

respectively, than for A549 cells, suggesting that gefitinib suppresses expression of HSP70 through an inhibitory effect on the self-phosphorylation of EGFR.

Activation of the EFGR affects various cellular responses through modulation of intracellular signal transduction, such as activation of JNK and ERK [45], [46]. To test whether inhibition of JNK or ERK is involved in gefitinib-dependent suppression of HSP70 expression, we examined the effect of inhibitors of JNK and ERK, SP600125 and U0126, respectively, on the expression of HSP70. Treatment of cells with SP600125 (Fig. 5B) but not U0126 (data not shown) decreased the level of HSP70, suggesting that the inhibition of JNK is involved in the gefitinib-dependent suppression of HSP70 expression.

Effect of gefitinib on bleomycin-induced pulmonary fibrosis

As described in the Introduction, we recently reported that expression of HSP70 protects against bleomycin-induced pulmonary fibrosis [35]. Therefore, the *in vitro* results outlined above imply that gefitinib exacerbates bleomycin-induced pulmonary fibrosis through suppression of HSP70 expression. To test this, we examined the effect of oral daily administration of gefitinib on the expression of HSP70 in the lungs. As shown in Fig. S2, this administration decreases the level of HSP70. The decrease became apparent at day 3 and was also observed at day 6 (Fig. S2). Therefore, to examine the effect of administration of gefitinib on bleomycin-induced pulmonary fibrosis, mice were orally administered gefitinib once per day for three days before receiving a single intratracheal administration of bleomycin; the administration of gefitinib was then continued, once every two days, for the following 14 days.

Histopathological analysis of pulmonary tissue using hematoxylin and eosin (H & E) staining revealed that the simultaneous oral administration of gefitinib (200 mg/kg) and intratracheal administration of bleomycin (1 mg/kg) produced severe pulmonary damage (thickened and edematous alveolar walls and interstitium, and infiltration of leucocytes) (Fig. 6A). Administration of either gefitinib or bleomycin alone did not cause such clear-cut pulmonary damage (Fig. 6A). Masson's trichrome staining of collagen revealed that administration of bleomycin caused slight collagen deposition, an effect that was greatly exacerbated by simultaneous administration of gefitinib (Fig. 6B). The pulmonary hydroxyproline level (an indicator of collagen levels) was increased by administration of bleomycin alone, and this effect was further

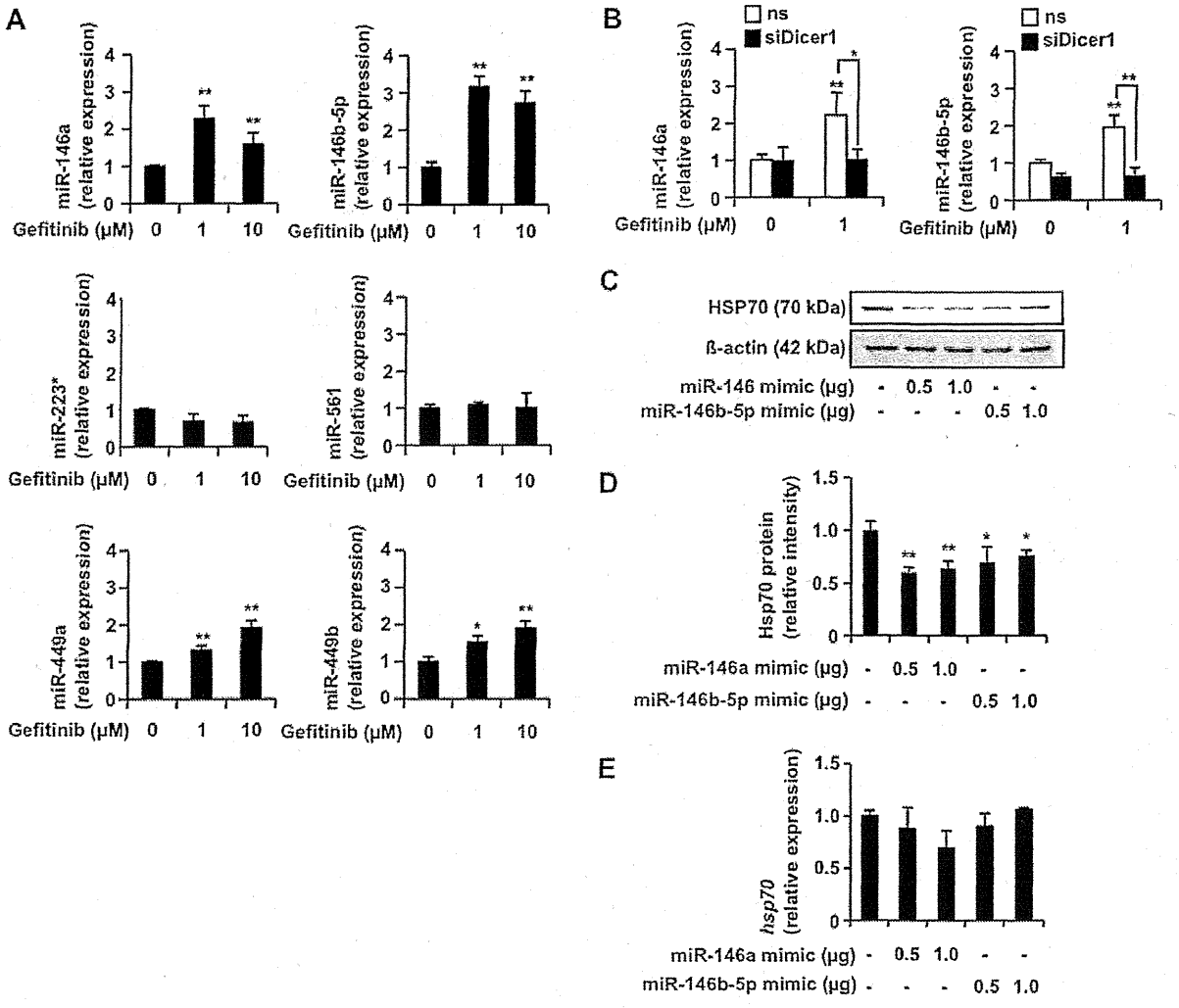


Figure 4. Identification of miRNAs involved in gefitinib-dependent suppression of expression of HSP70. A549 cells were incubated with the indicated concentration of gefitinib for 3 h (A). A549 cells were transfected with siRNA for Dicer1 (siDicer1) or non-specific siRNA (ns) and after 24 h were incubated with the indicated concentration of gefitinib for 3 h (B). A549 cells were transfected with the indicated amount (μg/well) of miRNA mimic RNA fragments for 24 h (C, E). The RNA (A, B, E) and protein (C) expression was monitored and expressed as described in the legend of Fig. 1. The RUN44 non-coding RNA was used for normalization (A, B, E). The intensities of the HSP70 bands were determined and are expressed relative to the control (one of the gels is shown in C) (D). Values shown are mean ± S.D. ($n=3$). $^{**}P<0.01$; $^{*}P<0.05$; n.s., not significant.
doi:10.1371/journal.pone.0027296.g004

enhanced by the simultaneous administration of gefitinib (Fig. 6E). Under our experimental conditions, administration of gefitinib alone did not cause collagen deposition nor increase the level of pulmonary hydroxyproline (Fig. 6B and E).

We also performed similar experiments in transgenic mice expressing HSP70. Since these mice are resistant to bleomycin, we used a higher dose (2 mg/kg) of bleomycin than in the previously described experiments. As shown in Fig. 6C, D and F, the gefitinib-dependent enhancement of bleomycin-induced pulmonary damage, collagen deposition and increase in pulmonary hydroxyproline level were not clearly observed in the transgenic mice, suggesting that gefitinib exacerbates bleomycin-induced pulmonary fibrosis through the suppression of HSP70 expression.

To test this idea, we examined the effect of the administration of gefitinib and/or bleomycin on pulmonary expression of HSP70 in wild-type mice and transgenic mice expressing HSP70. To begin with, we confirmed the overexpression of HSP70 in the lungs of transgenic mice in both the presence and absence of gefitinib administration (Fig. 6G). As shown in Fig. 6H and I, in wild-type mice, administration of gefitinib decreased the pulmonary level of HSP70 in both the presence and absence of simultaneous bleomycin administration. Administration of bleomycin alone also decreased the pulmonary level of HSP70 (Fig. 6H and I). In contrast, such a gefitinib-dependent decrease in the pulmonary level of HSP70 was not observed clearly in transgenic mice expressing HSP70 in both the presence or absence of simultaneous administration of bleomycin (Fig. 6J). We also found that using a lower dose of gefitinib (100 mg/kg) neither exacerbated bleomycin-induced pulmonary fibrosis nor suppressed pulmonary expression of HSP70 (Fig. S3). These results suggest that administration of gefitinib exacerbates bleomycin-induced pulmonary fibrosis through suppression of HSP70 expression.

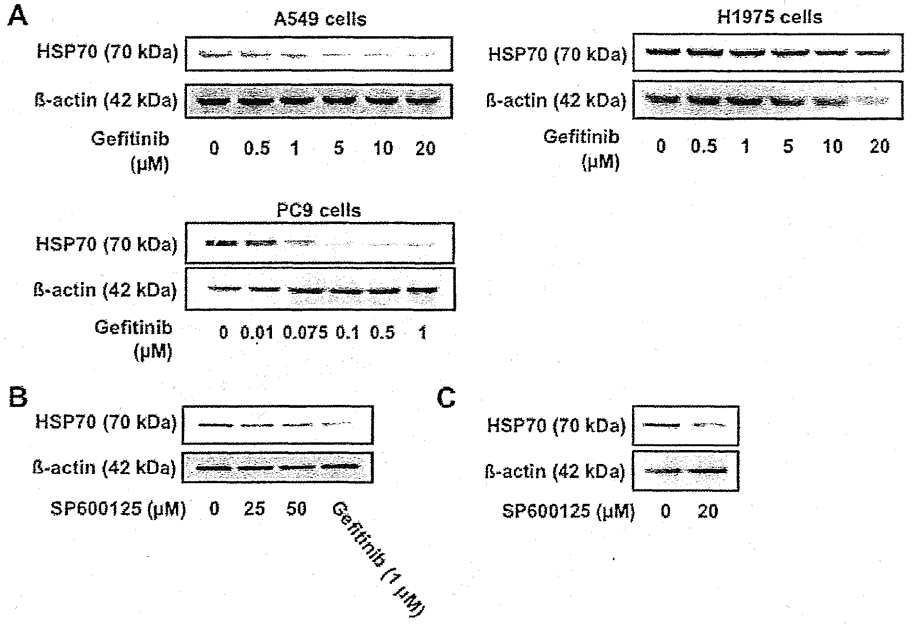


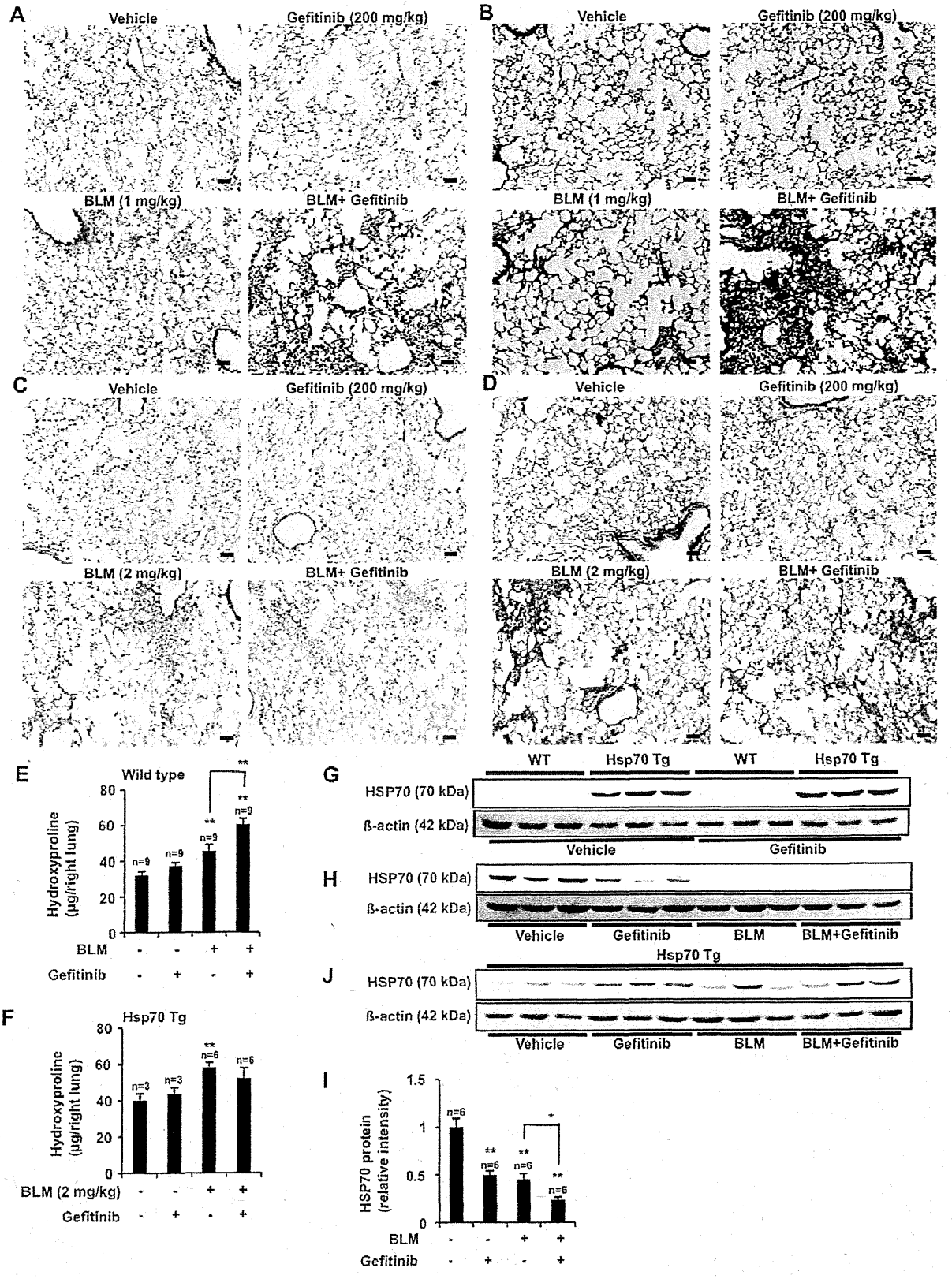
Figure 5. Involvement of inhibition of EGFR and JNK in gefitinib-dependent suppression of expression of HSP70. A549 (A-C) and H1975 and PC9 (A) cells were incubated with the indicated concentration of gefitinib (A, B) or SP600125 (B) for 24 h. Protein expression was monitored and is expressed as described in the legend of Fig. 1.
doi:10.1371/journal.pone.0027296.g005

For further confirmation of this idea, we examined the effect of co-administration of GGA. As shown in Fig. 7A-C, co-administration of GGA clearly suppressed the gefitinib-dependent enhancement of bleomycin-induced pulmonary tissue damage, collagen deposition and increases in the pulmonary hydroxyproline level in wild-type mice. We also examined the effect of GGA on the expression of HSP70 in the lung. As shown in Fig. 7D and E, co-administration of GGA recovered the gefitinib-suppressed expression of HSP70 in the lung, suggesting that GGA suppresses the gefitinib-dependent exacerbation of bleomycin-induced pulmonary fibrosis by inducing the recovery of HSP70 expression.

Discussion

Because the molecular mechanism governing drug-induced ILD (interstitial pneumonia associated with pulmonary fibrosis) is unknown, a suitable animal model is not available at present. Consequently, neither a clinical protocol for the treatment of drug-induced ILD nor an assay system to eliminate candidate drugs with the potential to cause this type of side effect has been established. We recently reported that A771726, an active metabolite of leflunomide, induced EMT of lung epithelial cells both *in vitro* and *in vivo*, and that administration of leflunomide exacerbated bleomycin-induced pulmonary fibrosis, suggesting that the leflunomide-dependent exacerbation of bleomycin-induced pulmonary fibrosis is mediated by the stimulation of EMT of lung epithelial cells. We proposed that examination of the EMT-inducing ability of candidate drugs is useful for screening to eliminate those with the potential side effect of inducing pulmonary fibrosis. We also found that leflunomide-dependent exacerbation of bleomycin-induced pulmonary fibrosis is ameliorated by the simultaneous intratracheal administration of uridine, which suppresses the A771726-dependent induction of EMT *in vitro*, and propose that this administration is beneficial for the treatment of leflunomide-induced pulmonary fibrosis in humans. In the present study, we followed a similar strategy, focusing on the expression of HSP70, because we had recently reported that expression of HSP70 protects against bleomycin-induced pulmonary fibrosis through cytoprotective and anti-inflammatory effects and, by inhibiting the production of TGF-β1 and TGF-β1-dependent EMT of lung epithelial cells [35].

We have found that gefitinib suppresses the expression of HSP70 *in vitro*. The concentration of gefitinib used (1 μM) is similar to that obtained in plasma when administered at therapeutic levels (about 1-2 μM) [47]. Thus, we considered that the suppression of HSP70 expression by gefitinib is clinically relevant and investigated the molecular mechanism underlying this effect. We concluded that treatment of cells with gefitinib (1 μM) decreases the level of HSP70 through inhibition of translation based on observations that (i) the gefitinib-dependent decrease in the level of HSP70 was suppressed by an inhibitor of translation, (ii) the translation of HSP70, measured by pulse-labelling experiments, was inhibited by treatment of cells with gefitinib, and (iii) *hsp70* promoter activity, the level and stability of *hsp70* mRNA and the stability of HSP70 were all unaffected by treatment of cells with gefitinib. Furthermore, we suggested that two miRNAs (miR-146a and miR-146b-5p) are involved in this gefitinib-dependent suppression of HSP70 translation; this was based on our observations that (i) the introduction of the 3' UTR of *hsp70* into a luciferase reporter plasmid caused a gefitinib-dependent decrease in luciferase activity, (ii) the gefitinib-dependent suppression of HSP70 translation was not observed in cells transfected with siRNA for Dicer1, and (iii) gefitinib increased the levels of miR-146a and miR-146b-5p, both of which have the ability to decrease the level of HSP70. Since for various cancer cells and tissues, down-regulation of expression of these miRNAs and up-regulation of HSP70 and the contribution of these to cancer progression have been reported [48], [49], [50], the results


A
Vehicle
Gefitinib (200 mg/kg)
BLM (1 mg/kg)
BLM+ Gefitinib
B
Vehicle
Gefitinib (200 mg/kg)
BLM (1 mg/kg)
BLM+ Gefitinib
C
Vehicle
Gefitinib (200 mg/kg)
BLM (2 mg/kg)
BLM+ Gefitinib
D
Vehicle
Gefitinib (200 mg/kg)
BLM (2 mg/kg)
BLM+ Gefitinib
E
Wild type
Hydroxyproline (μg/right lung)
BLM
Gefitinib
F
Hsp70 Tg
Hydroxyproline (μg/right lung)
BLM (2 mg/kg)
Gefitinib
G
WT
Hsp70 Tg
WT
Hsp70 Tg
HSP70 (70 kDa)
ß-actin (42 kDa)
Vehicle
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Vehicle
Gefitinib
BLM
BLM+Gefitinib
J
Hsp70 Tg
HSP70 (70 kDa)
ß-actin (42 kDa)
Vehicle
Gefitinib
BLM
BLM+Gefitinib
I
HSP70 protein (relative intensity)
BLM
Gefitinib

Figure 6. Effect of gefitinib on bleomycin-induced pulmonary fibrosis and pulmonary expression of HSP70. Wild-type mice (A, B, E, G, H) and transgenic mice expressing HSP70 (C, D, F, G, J) were orally administered gefitinib (200 mg/kg) or vehicle once per day for 3 days (from day 0 to day 2), then treated once only with or without 1 mg/kg (for wild-type mice) or 2 mg/kg (for the transgenic mice) bleomycin (BLM) (day 3). Mice were then orally administered gefitinib (200 mg/kg) or vehicle once per 2 days for 14 days (from day 3 to day 17). Sections of pulmonary tissue were prepared at day 17 and subjected to histological examination (H & E staining (A, C) or Masson's trichrome staining (B, D)) (scale bar, 50 μm) (A-D). Pulmonary hydroxyproline levels at day 17 were determined (E, F). Total protein was extracted from pulmonary tissues at day 17 and analyzed by immunoblotting with an antibody against HSP70 or actin (G, H, J). The intensities of the HSP70 bands were determined (one of the gels is shown in H) and are expressed relative to the control (I). Values are mean ± S.E.M. *P<0.05; **P<0.01; n.s., not significant.
doi:10.1371/journal.pone.0027296.g006

of this study suggest the latter up-regulation is caused by the former down-regulation.

A decrease in the level of HSP70 was also observed in cells treated with 10 μM gefitinib; however, the mechanism governing this decrease seems to be different from that seen with 1 μM gefitinib, because treatment of cells with 10 μM gefitinib caused a decrease in the level of *hsp70* mRNA and in the stability of both *hsp70* mRNA and HSP70. We consider that this high con-

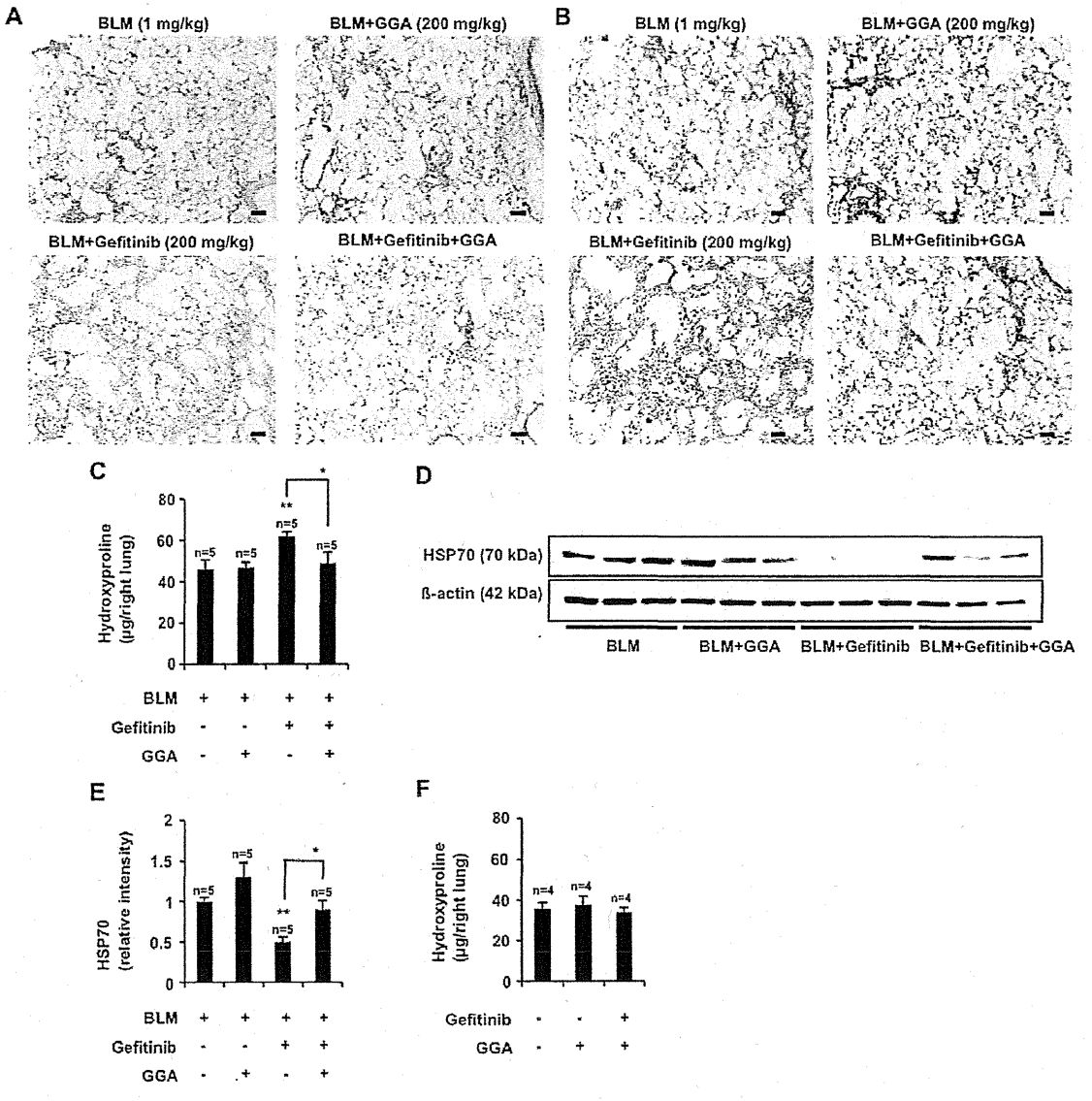

Figure 7. Effect of GGA on gefitinib-dependent exacerbation of pulmonary fibrosis and pulmonary expression of HSP70. Wild-type mice were orally administered gefitinib (200 mg/kg) and/or GGA (200 mg/kg) once per day for 3 days (from day 0 to day 2), then treated once only with 1 mg/kg bleomycin (BLM) (day 3). Mice were then orally administered gefitinib (200 mg/kg) and/or GGA (200 mg/kg) once per 2 days for 14 days (from day 3 to day 17). Sections of pulmonary tissue were prepared at day 17 and subjected to histological examination (H & E staining (A) or Masson's trichrome staining (B)) (scale bar, 50 μm). The pulmonary hydroxyproline levels were determined at day 17 (C). Total protein was extracted from pulmonary tissues at day 17 and analyzed and expressed as described in the legend of Fig. 6 (D, E). Values are mean ± S.E.M. **P<0.01; *P<0.05.
doi:10.1371/journal.pone.0027296.g007

centration of gefitinib pleiotropically affects various cellular reactions.

After binding EGF, the EGFR is self-phosphorylated and transduces the signal via various mechanisms, including the activation of JNK. Here, we have found that mutations in the EGFR that alter the concentration of gefitinib required for inhibition of EGFR self-phosphorylation affect the gefitinib-dependent decrease in the level of HSP70. Furthermore, an inhibitor of JNK caused a decrease in the level of HSP70. These results suggest that inhibition of EGFR self-phosphorylation and the resulting inhibition of JNK are involved in the gefitinib-dependent decrease in the level of HSP70. However, the manner by which inhibition of JNK suppresses translation of HSP70 remains unclear.

Administration of gefitinib alone did not produce pulmonary fibrosis under our experimental conditions. As one of the risk factors for gefitinib-induced ILD is pre-existing pulmonary fibrosis, we hypothesized that gefitinib stimulates pulmonary fibrosis in the presence of other fibrosis-inducing stimuli. In fact, we found that administration of gefitinib stimulates bleomycin-induced pulmonary fibrosis. Interestingly, this gefitinib-dependent stimulation was not observed so clearly in transgenic mice expressing HSP70, suggesting that expression of HSP70 is involved in the gefitinib-induced exacerbation of bleomycin-induced pulmonary fibrosis. We found that administration of gefitinib suppressed the pulmonary expression of HSP70 and that this suppression was not observed in the transgenic mice expressing HSP70. Taken together, our findings suggest that gefitinib exacerbates bleomycin-induced pulmonary fibrosis through the suppression of HSP70 expression. This finding is an important step towards elucidating the molecular mechanism of drug-induced ILD, as well as the mechanism governing the ethnic differences in susceptibility to ILD induced by the drug. It is possible that the susceptible phenotype of Japanese patients is due to a specific polymorphism in the *hsp70* and *hsf1* genes and in other genes related to HSP70. Also, examination of the effect of candidate drugs on the expression of HSP70 *in vitro* could be used for screening to eliminate those drugs with the potential to induce ILD. Furthermore, exacerbation of bleomycin-induced pulmonary fibrosis was observed with leflunomide [7] and gefitinib (this study), suggesting this model can be used as an animal model of drug-induced pulmonary fibrosis and for screening to eliminate candidate drugs with the potential to induce ILD.

As described above, it was recently reported that administration of GGA ameliorates bleomycin-induced pulmonary fibrosis [51], a result that we confirmed in a previous paper [35], In this study, we found that administration of GGA suppresses the gefitinib-dependent exacerbation of bleomycin-induced pulmonary fibrosis. We consider that this suppression is due to the recovery of HSP70 expression in the lung. As described in the Introduction, a treatment for drug-induced ILD has not been established. We believe that GGA could be beneficial for the treatment of gefitinib-induced ILD, given that the safety of GGA has already been shown clinically.

Supporting Information

Figure S1 Effects of drugs known to induce ILD clinically on expression of HSP70. A549 cells were incubated with the indicated concentration of A771726, amiodarone (AMD), gefitinib or imatinib for 24 h. Whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or β-actin.
(TIFF)

Figure S2 Time course profile for gefitinib-dependent suppression of expression of HSP70 *in vivo*. Mice were orally administered gefitinib (200 mg/kg) or vehicle once per day for the indicated periods. Total protein was extracted from pulmonary tissues and protein expression was monitored by immunoblotting with an antibody against HSP70 or β-actin.
(TIFF)

Figure S3 Effect of low dose of gefitinib on bleomycin-induced pulmonary fibrosis and pulmonary expression of HSP70. The effect of a low dose of gefitinib (100 mg/kg) on the expression of HSP70 in the lung (A) and pulmonary hydroxyproline levels (B) were monitored as described in the legend of Fig. 6. Values are mean ± S.E.M.
(TIFF)

Author Contributions

Performed the experiments: TN TH TM. Analyzed the data: TN KT TM. Contributed reagents/materials/analysis tools: TN AA TM. Wrote the paper: TN TM.

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