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Stimulation of gastric ulcer healing by heat shock protein 70

Tomoaki Ishihara, Shintaro Suemasu, Teita Asano, Ken-ichiro Tanaka, Tohru Mizushima *

Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan

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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.

* Corresponding author. Tel.: +81 3 5400 2628; fax: +81 3 5400 2628.
E-mail addresses: mizushima-th@pha.keio.ac.jp, mizu@gpo.kumamoto-u.ac.jp (T. Mizushima).

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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,