Heat shock protein 27: Induction by gastroduodenal reflux in vivo and augmentation of human esophageal mucosal cell growth in vitro

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Objective: Acid exposure to esophageal epithelium leads to hyperplasia and mucosal thickening. This is associated with upregulation of antiapoptotic genes. Recently, heat shock proteins have been implicated in esophageal mucosal response to stress. We sought to determine the influence of gastroduodenal reflux on esophageal mucosal heat shock protein 27 gene (murine analog Hspb1, human HSPB1) expression in vivo and the effect of HSPB1 overexpression on proliferation of esophageal mucosal cells in vitro.

Methods: Balb/c mice underwent either anastomosis of gastroesophageal junction and first portion of duodenum to induce continuous gastroduodenal reflux (n = 14) or sham procedure (n = 12). Quantitative reverse transcriptase polymerase chain reaction was used to determine the influence of gastroduodenal reflux on Hspb1 expression. Immunofluorescent microscopy and immunoblotting were used to quantify changes in heat shock protein 27 protein expression. Lentiviral infection techniques were used to overexpress HSPB1 in human esophageal epithelial cells. Both 3-(4,5-dimethylthiazole-2-yl) 2,5,-diphenyl tetrazolium bromide and 5-bromo-2-deoxyuridine incorporation assays were used to assess cell proliferation.

Results: Expressions of Hspb1 and its protein product were increased in esophageal tissue after 12 weeks’ reflux relative to sham control group. Expression was located mainly in hyperplastic epithelial cells. Overexpression of HSPB1 in human esophageal epithelial cells resulted in increased proliferation.

Conclusions: Heat shock protein 27 is upregulated in response to gastroduodenal reflux and is a mediator of human esophageal epithelial cell proliferation and growth. This novel finding illustrates the importance of its expression in the development of inflammation and mucosal thickening associated with esophageal reflux.

Gastroesophageal reflux disease (GERD) is estimated to affect 19 million people in the United States, with associated costs of $24 billion per year. The distribution of these costs includes 25% for drug costs, 15% for health care costs, and 60% in lost productivity.1 Serious complications of GERD include esophageal stricture, Barrett esophagus, and esophageal adenocarcinoma.1,2 The main element in the development of these problems related to GERD is chronic inflammation of the esophageal mucosa.3,4 The pathologic lesion produced by GERD is one of hyperplasia and mucosal thickening, which indicates that abnormal growth regulation in the esophageal mucosa may be the underlying cause. Known growth regulatory proteins, such as epidermal growth factor and transforming growth factor α have been shown to be upregulated in esophageal mucosa exposed to GERD.5 Likewise, acid exposure of esophageal mucosa appears to upregulate several antiapoptotic and antioxidant-associated genes.6 Our current understanding of the growth response in esophageal mucosa is minimal, and identification of new potential mediators of this response is critical.

Recently, heat shock proteins (HSPs) have been implicated in the esophageal mucosal response to acidic stress.7 HSPs are a set of evolutionarily conserved molecular chaperone proteins that are induced by various factors, such as thermal, oxidative, and inflammatory stressors.8 The main function of HSPs in these stress responses is to protect cells against apoptosis and death. This subsequent dysregulation and inhibition of apoptosis is what may link HSPs to the altered growth seen in the hyperplastic esophageal mucosa in response to GERD and potentially to the carcinogenesis of esophageal malignancy. Raifee and colleagues9 have demonstrated that HSP27 levels are induced in vitro in human esophageal microvascular endothelial cells in response to acidic stress. HSP27 has also been detected in esophageal carcinomas, indicating a potential role in the pathogenesis of this disease.

We have observed in a murine model reproducible changes in the esophageal mucosa identical to the hyperplasia and mucosal thickening seen in human esophageal reflux.10 Our aim in this study was to determine whether
Abbreviations and Acronyms

AKT = protein kinase B
BrdU = bromodeoxyuridine
GDR = gastroduodenal reflux
GERD = gastroesophageal reflux disease
HSP = heat shock protein
Hspb1 = murine gene for heat shock protein 25
HSPB1 = human gene for heat shock protein 27
MTT = 3-(4,5-dimethylthiazole-2-yl) 2,5-diphenyl tetrazolium bromide
PBS = phosphate-buffered saline solution
PCR = polymerase chain reaction

HSP27 (or in this case the murine analog, HSP25) is upregulated in response to gastroduodenal reflux in vivo, as well as to determine whether overexpression of HSP27 alters the growth potential of human esophageal mucosal cells in vitro.

MATERIALS AND METHODS
Animal Model

Male Balb/c mice (Jackson Labs, Bar Harbor, Me) 8 to 12 weeks old were fed a standard diet (Harlan Teklad 2018; Harlan Laboratories, Inc, Madison, Wis) and water ad lib. Animals were allowed to acclimatize for 7 days before surgery. Animals were kept fasting for 12 hours before surgery but were allowed access to water. The Animal Care and Use Committee at the University of Colorado at Denver Health Sciences Center approved all experiments, and this investigation conformed to the Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog/5140.html). Duodenogastroesophageal anastomosis was performed (n = 142) through a midline laparatomy to create a 7-mm side-to-side anastomosis with 10-0 nylon suture, as previously described.10 The abdomen was irrigated with warm saline solution, and the laparotomy was closed. This operation created continuous mixed reflux onto the esophageal epithelium just above the gastroesophageal junction. The sham procedure consisted of laparotomy and esophagotomy just above the gastroesophageal junction, followed by closure of both the esophagotomy and laparotomy (n = 12). There were no deaths in the sham group. All animals were humanely killed with carbon dioxide inhalation. The 12 mice in each group were divided into groups of 4 for each experimental medium.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

The distal 0.5 cm of each esophagus (n = 4) was removed and immediately placed in RNAlater solution (Qiagen Inc., Valencia, Calif). The tissue was then homogenized, and total RNA was extracted with the RNeasy Protect Kit (Qiagen). Complementary DNA was synthesized from 5 μg of total RNA with SuperScript II Reverse Transcriptase, Random Primers, and dNTP mix (Invitrogen Corporation, Carlsbad, Calif). Quantitative polymerase chain reaction (PCR) was carried out with the Rotor-Gene 6000 and dNTP mix (Invitrogen Corporation, Carlsbad, Calif). Quantitative polymerase chain reaction (PCR) was carried out with the Rotor-Gene 6000 and dNTP mix (Invitrogen Corporation, Carlsbad, Calif). Reverse transcriptase polymerase chain reaction was performed with the Primer 3 program. The sequence of the forward primer was GAGATCACTGGCAAGCACGA; that of the reverse was TGGAGGGAGCGTGTATTTCC. The forward primer for the gene for glyceraldehyde 3-phosphate dehydrogenase was GAGTCAACGGATTGTTGGTGT; that of the reverse was GACAAGCTCCCCGTTCCTCAG. All reactions were performed in a total volume of 20 μL with 20 ng complementary DNA (based on the original RNA concentration) and 0.5-μg concentrations of the forward and reverse primers. Negative (no added template) controls were run for each set of experiments. PCR reactions were set at 95°C for 5 minutes, followed by 40 cycles including a denaturation step at 95°C for 5 seconds, an annealing step at 63°C for 10 seconds, and an extension step at 72°C for 15 seconds. The last step included a slow increase in temperature from 72°C to 95°C, or melt curve. Verification of the appropriate product was conducted by melt curve analysis as well as agarose gel electrophoresis. Data was calculated as relative abundance of Hspb1 messenger RNA (mRNA) relative to β-2-mRNA of the gene for glyceraldehyde 3-phosphate dehydrogenase according to the ∆∆Ct method.

Immunofluorescent Staining

After mobilization, the entire esophagus was flushed with Tissue Freezing Medium (Triangle Biomedical Sciences, Inc, Durham, NC). The distal 5 mm was then embedded in Tissue Freezing Medium and frozen in an orientation that allowed axial sectioning of the esophageal lumen (n = 4). Sham control esophagus was prepared in exactly the same manner (n = 4). The most distal portion of each sample was used for comparison in each animal.

Cryosections (5 μm thick) were cut with a cryostat (IEC Minomite Plus; International Equipment Co, Needham Heights, Mass) and collected on poly-L-lysine–coated slides. Sections were treated with a mixture of 70% acetone and 30% methanol for 5 minutes and then fixed with 4% parafomaldehyde for 10 minutes. Sections were washed with phosphate-buffered saline solution (PBS), blocked with 10% normal serum for 30 minutes, and incubated overnight with a polyclonal rabbit ant–mouse HSP25 antibody (Stressgen; Assay Designs, Inc, Ann Arbor, Mich) at 5 μg/mL in PBS containing 1% bovine serum albumin antibody. After washing with PBS, sections were incubated with Cy3-conjugated matched IgG (Jackson Immunoresearch Laboratories, Inc, West Grove, Pa) at 1:100 dilution with PBS containing 1% bovine serum albumin. To assess specificity, adjacent sections were incubated with non–immune-matched IgG (5 μg/mL in PBS containing 1% bovine serum albumin) and otherwise processed identically. Primary incubation was performed at 4°C, and all other incubations were performed at room temperature. To stain nuclei, bis-benzimidize was used (4’,6-diamidino-2-phenylindole, imaged on the blue channel) and wheat-germ agglutinin to stain cell membranes (labeled with Alexa 488 and imaged on the green channel). HSP25 was imaged using the red (Cy3) channel. Microscopic observation and photography were performed with a Leica DMRXA confocal microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany).

Image Quantitation

Images were quantitated with SlideBook version 4.0 software (Intelligent Imaging Innovations, Inc, Denver, Colo). Four random images focusing on the esophageal epithelium were taken from each section at 40× magnification. All images were taken while blinded to the Cy3 channel. Images were masked to exclude 95% of nonspecific fluorescence, as determined from images of negative controls. SlideBook was then used to determine the area (in square micrometers) of positive staining, as well as mean fluorescence intensity of staining. Mean area and intensity per field were calculated for each section.

Immunoblotting

The distal 1 cm of esophagus above the anastomosis was removed and flash frozen in liquid nitrogen (n = 4 for both reflux and sham groups). The specimen was then stored at –80°C until further use. When ready for use, tissue was homogenized in mammalian cell lysis buffer (Roche Diagnostics GmbH Mannheim, Mannheim, Germany) and protein content was determined from images of negative controls. SlideBook was then used to determine the area (in square micrometers) of positive staining, as well as mean fluorescence intensity of staining. Mean area and intensity per field were calculated for each section.

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determined with a bicinchoninic acid protein assay (Thermo Fisher Scientific Inc, Rockford, Ill). Approximately 30 μg protein was loaded per lane of a 4% to 20% gradient gel (Bio-Rad Laboratories, Inc, Hercules, Calif), and membranes were run in tris(hydroxymethyl)aminomethane-glycine running buffer. Protein from gels was then transferred to a nitrocellulose membrane and blocked in 5% nonfat milk for 1 hour. The membrane was then incubated in 1:1000 rabbit anti–mouse HSP25 antibody (Stressgen) overnight at 4°C. After primary incubation, membranes were washed 3 times for 10 minutes each in PBS-polysorbate. Membranes were then incubated at room temperature in 1:5000 horseradish peroxidase–linked secondary antibodies for 1 hour. Membranes were then rinsed with PBS-polysorbate, and target bands were developed with enhanced chemiluminescence and exposed on film.

**RESULTS**

Outcomes of Surgical Procedures

In the course of 26 performed operations, there were 2 deaths in the immediate perioperative period, both of animals that had undergone the reflux procedure. There were no other deaths among the remaining mice, leaving 12 mice each in the reflux and sham groups. All animals had similar weight gain and appeared grossly normal at the termination of the experiment. All anastomoses were determined to be patent by probing with forceps during tissue harvest.

**Hspb1 Gene Expression Induction by Mixed Reflux**

Expression of Hspb1 (murine analog to HSPB1) mRNA in esophageal tissue exposed to GDR (n = 4 in each group) was determined with real-time reverse transcriptase PCR and compared with expression in the sham control group (n = 4). After 12 weeks of exposure to GDR, there was a greater than 2-fold increase (P < .01) in Hspb1 mRNA relative to the sham control group (Figure 1).

HSP25 Protein Level Increase in Response to Mixed Reflux

There was minimal staining for HSP25 in the sham control group. In contrast, animals that had undergone reflux surgery had significant induction of the enzyme, particularly in the epithelium. After 12 weeks of GDR, there was a greater than 3-fold increase in area of positive staining in the esophageal epithelium and a greater than 2-fold increase in fluorescent intensity of staining (Figure 2). Immunoblot of tissue homogenates also showed a 75% increase in HSP25...
protein after normalization to glyceraldehyde 3-phosphate dehydrogenase (Figure 3).

**Overexpression of HSP27 and Augmented Growth of Esophageal Epithelial Cells**

Het-1A cells were efficiently infected with LVUT lentiviral particles without affecting the health of the cells (Figure 4, A). Immunoblotting showed overexpression of HSP27 in the cells infected with LVUT-HSPB1 relative to the control group (Figure 4, B). The HSPB1 transgene was slightly larger because of the addition of proline residues in the transgene so that the expression of the transgene could be distinguished from endogenous expression. Overexpression of HSP27 in Het-1A cells led to a greater than 20% increase in cell number according to MTT assay and a greater than 30% increase in proliferation according to BrdU-incorporation assay (Figure 5).

**DISCUSSION**

HSP27 expression is associated with inflammatory conditions of the gastrointestinal tract, such as acute and chronic pancreatitis and inflammatory bowel disease. The
expression pattern of this protein in reflux esophagitis, however, is unknown. In this study, we used a well-established murine model of mixed reflux to show that expression of HSP25 (the murine analog of HSP27) is upregulated in esophageal tissue in vivo. We also showed that most of this upregulation occurs in the esophageal epithelial cells. Furthermore, we demonstrated that overexpression of HSP27 in human esophageal epithelial cells leads to an increase in cellular proliferation and growth.

One of the earliest histologic responses of the esophageal epithelium to exposure to gastric and duodenal reflux is hyperplasia with mucosal thickening. These cellular changes are thought to represent a protective response to acidic stress and are mediated by growth factors, cytokines, and reactive oxygen species. Hyperplasia is also thought to characterize the first step in the inflammation, metaplasia, dysplasia, adenocarcinoma sequence that is associated with long-standing reflux disease. The mechanisms involved in the transition from epithelial hyperplasia to metaplasia are poorly understood. Because the incidence of esophageal adenocarcinoma has risen dramatically during the last 3 decades, and survival with this devastating disease remains poor, identification of proteins involved in this process is important.

HSPs are molecular chaperone proteins that are upregulated in response to stressful stimuli such as heat, reactive oxygen species, and acidic pH. One of their functions is to

FIGURE 3. Immunoblot quantification of heat shock protein 27 analog expression. Esophageal tissue exposed to GDR has 75% more heat shock protein 25 (HSP25, murine analog of heat shock protein 27) than sham control after normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; n = 4 in each group). Asterisk indicates P < .01.

FIGURE 4. Lentiviral overexpression of heat shock protein 27 (Hsp27) in esophageal epithelial cells. A, Lentiviral infection is effective for overexpressing protein in Het-1A cells, as shown by transfection with vector containing green fluorescent protein gene. B, Het-1A cells infected with vector containing gene for heat shock protein 27 (LVUTHsp27) contain higher heat shock protein 27 levels than empty vector controls (LVUT) or untreated controls (UT). GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.
prevent cells from undergoing apoptosis when stressed by such stimuli. They accomplish this by preventing denaturation of key intracellular proteins, stabilizing actin filaments, and inhibiting key enzymes involved in the apoptotic process. HSPs are characterized by molecular size and are constitutively expressed in a broad range of normal tissues.

We have identified HSP27 as a potential mediator of the hyperplastic response to mixed reflux in esophageal epithelium. After 12 weeks of exposure to surgically induced mixed reflux, Balb/c mice develop epithelial hyperplasia and mucosal thickening that mimics the changes seen in human beings. With this murine model, we have found that Hsp1b, the murine analog to HSP1B, is upregulated at both the mRNA and protein levels in esophageal tissue. With immunofluorescent microscopy, we identified the hyperplastic epithelium as the area of greatest protein expression. Soldes and colleagues demonstrated that HSP27 is expressed in normal human squamous esophageal epithelium and also that its expression is reduced significantly in Barrett metaplasia and esophageal adenocarcinoma. These authors went on to show that immortalized esophageal epithelial cells have a greater induction of HSP27 expression in response to heat shock than do esophageal adenocarcinoma cells. These findings suggest that HSP27 may not be an important mediator of cell growth in metaplastic or malignant cells; however, its overexpression in chronically inflamed esophageal mucosa may assist in the development of epithelial hyperplasia by preventing cell death. Prolonging the life of stressed esophageal epithelium could lead to the accumulation of genetic mutations necessary for the transition to Barrett esophagus or adenocarcinoma. It is known that the pathways regulating cell cycle and differentiation differ considerably between squamous and metaplastic or malignant cells. This could explain the discrepancy found by Soldes and colleagues. Further studies are needed to determine the role of HSP27 in esophageal metaplasia and adenocarcinoma.

The epithelial cell is not the only esophageal cell type that upregulates HSP27 in response to reflux. Rafiee and associates showed that HSP27 could be induced by acidic stress in primary human esophageal microvascular endothelial cells and that this induction was dependent on activation of p38 mitogen-activated protein kinase and protein kinase B (AKT). These authors concluded that HSP27 upregulation in the esophageal microvasculature likely plays an important role in the pathophysiologic mechanisms of reflux.

After recognizing that HSP27 protein expression is upregulated in hyperplastic esophageal epithelium in vivo, we wanted to determine the role which HSP27 plays in proliferation in vitro. We used lentiviral infection techniques to overexpress HSP27 in immortalized human esophageal epithelial (Het-1A) cells. With both MTT and BrdU-incorporation assays, we found that overexpression of HSP27 led to a significant increase in proliferation relative to empty vector and untreated controls. To our knowledge, this is the first report of HSP27 mediating proliferation in esophageal epithelium.

HSP27 is known to modulate cell cycle through the activation of AKT, a protein kinase implicated in both proliferation and apoptosis, in many different cell types. There are considerable data to suggest that AKT is an important mediator of proliferation in esophageal disease. Beales and coworkers found that the active phosphorylated form of AKT was upregulated in esophageal adenocarcinoma both in vitro and in vivo. Furthermore, these authors were able to inhibit acid-induced proliferation in esophageal adenocarcinoma cells by inhibiting the phosphorylation of AKT. Oyama and associates demonstrated the importance of AKT in primary esophageal epithelial cell proliferation. With organotypic culture, these authors were able to replicate the hyperplastic phenotype of reflux esophagitis by overexpressing the epidermal growth factor receptor. When these cells were treated with an inhibitor that prevented phosphorylation of AKT, the cells lost their hyperplastic phenotype and reverted to monolayer growth. In light of these data, one might speculate that the increase in proliferation we found in esophageal

![FIGURE 5](image-url)
epithelial cells overexpressing HSP27 occurred through the activation of AKT. Further studies are needed to answer this question.

In summary, the results of this study indicate that the expression of HSP25, the murine analog of HSP27, is upregulated by esophageal epithelium in vivo in response to reflux. Furthermore, overexpression of HSP27 in human esophageal epithelial cells results in an increase in proliferation in vitro. HSP27 has been identified as a possible target for the prevention of abnormal growth and proliferation of esophageal epithelium associated with reflux disease.

References


