Antibodies to human gastric epithelial cells and heat shock protein 60 in *Helicobacter pylori* positive mucosa associated lymphoid tissue lymphoma

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Abstract

Background—Development of gastric mucosa associated lymphoid tissue (MALT) lymphoma is thought to be closely associated with host immune reactions to *Helicobacter pylori*.

Aim—To investigate humoral immune responses in patients with MALT lymphoma to antigens shared by *H pylori* and human gastric epithelial cells.

Methods—Sera were obtained from *H pylori* positive patients with MALT lymphoma (n = 11) or other gastroduodenal diseases (peptic ulcer, n = 40; non-ulcer dyspepsia, n = 20) and from *H pylori* negative healthy control subjects (n = 10). Antibodies to HGC-27 human gastric epithelial cells and human recombinant heat shock protein (Hsp) 60 were examined using an enzyme linked immunosorbent assay (ELISA) and immunoblotting.

Results—Antibody titres to HGC-27 cells were significantly elevated in *H pylori* positive patients with MALT lymphoma when compared with titres in patients with other gastroduodenal diseases and in healthy subjects. Immunoblotting of sera from patients with MALT lymphoma often detected a band with a molecular mass corresponding to Hsp60, and both ELISA and immunoblotting showed elevated antibody titres to the recombinant human Hsp60. Antigenic similarity between Hsp60 and *H pylori* HspB was documented by immunoblotting experiments. **Conclusions**—Autoantibodies reactive with host gastric epithelial cells are often increased in MALT lymphoma, and Hsp60 is a major target antigen. Immune responses induced by immunological cross reactivity between *H pylori* HspB and human Hsp60 in gastric epithelium may be involved in the development of MALT lymphoma.

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Accumulating evidence suggests that *Helicobacter pylori* is involved in the pathogenesis of gastric lymphoma of mucosa associated lymphoid tissue (MALT) type.¹⁻⁴ Antigenic mimicry between *H pylori* and the host mucosa⁵ may induce host autoimmune responses which

lead to the development of the disease. Heat shock protein (Hsp) is one candidate for the cross reacting antigen(s).⁶ Hsps are a family of proteins induced by cell stress, such as microbial infections, and are conserved in both prokaryotic and eukaryotic cells. Hsps with identical antigenic structures have been shown by immunohistochemistry in *H pylori* bacterial cells and gastric epithelial cells of patients infected with *H pylori*.⁷ In this study, we investigated host humoral immune responses to *H pylori* which may lead to the production of autoreactive antibodies. We measured antibodies against gastric epithelial cells and human Hsp60 in patients with MALT lymphoma, peptic ulcers, and non-ulcer dyspepsia using an enzyme linked immunosorbent assay (ELISA) and immunoblotting.

Patients and methods

PATIENTS

Sera were obtained from 71 *H pylori* positive patients, including 11 with MALT lymphoma (six women and five men; mean age 59.8 years), 20 with gastric ulcer (three women and 17 men; mean age 46.3 years), 20 with duodenal ulcer (10 women and 10 men; mean age 30.4 years), and 20 with non-ulcer dyspepsia (six women and 14 men; mean age 41.5 years). Sera were also obtained from 10 healthy volunteers (seven women and three men; mean age 37.9 years old). Informed consent was obtained from each patient and healthy volunteer.

Diagnosis was made based on findings of endoscopic examination and histological examination of gastric biopsy or gastrectomy specimens. Histology of MALT lymphoma was assessed according to the REAL classification.⁸ Tissue specimens were analysed immunohistochemically using antibodies to Igκ, Igλ, CD20, CD3, CD75, and bcl-2 (Dako Japan, Tokyo, Japan). Immunoglobulin heavy chain gene rearrangement was examined by Southern blotting and hybridisation with a probe to the joining region of the immunoglobulin heavy chain gene.⁹ All the lymphomas showed lymphoepithelial lesions histologically and an immunophenotype compatible with that of MALT lymphoma. Monoclonality of the tumours was shown either by light chain restriction immunohistochemically or by molecular

Abbreviations used in this paper: MALT, mucosa associated lymphoid tissue; Hsp, heat shock protein; ELISA, enzyme linked immunosorbent assay; PBS, phosphate buffered saline.

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Figure 1 Serum antibody titres to HGC-27 cells in patients with mucosa associated lymphoid tissue mucosa associated lymphoid tissue (MALT) lymphoma, gastric ulcer, duodenal ulcer, or non-ulcer dyspepsia, and control healthy subjects. Serum antibodies to HGC-27 human gastric epithelial cells were measured using cell ELISA with plates seeded with HGC-27 cells. *** $p < 0.001$ (Student's *t* test).

genetic examination. All the lymphomas were low grade.

Infection with *H pylori* was documented by culture, rapid urease test, and histology of gastric biopsy specimens and by the presence of serum antibodies to *H pylori* by ELISA.¹⁰ *H pylori* was detected in the gastric mucosa of all patients whose sera were positive for anti-*H pylori* antibodies. Negative *H pylori* infection of the control healthy volunteers was defined by seronegativity to *H pylori*.

ELISA FOR THE DETECTION OF ANTIBODIES TO HUMAN GASTRIC EPITHELIAL CELLS AND HUMAN HSP60

Serum antibodies to gastric epithelial cells were measured by cell ELISA. HGC-27 human gastric cancer cells¹¹ were cultured in 96-well microtitre plates. The cells were washed once gently with phosphate buffered saline (PBS), fixed with 2% formalin in PBS for two hours at room temperature, and washed twice with PBS. After blocking with PBS containing 10% skimmed milk, plates were incubated with 1:100 diluted serum for two hours, and washed with PBS containing 0.05% Tween 20. Then peroxidase labelled rabbit anti-human IgG antibody (Dako Japan) was added, and the plates were incubated for two hours. After being washed, the well contents were allowed to react with *o*-phenylenediamine in citrate buffer, pH 5.5. Absorbance at 490 nm was measured by an ELISA plate reader.

For the detection of antibodies to human Hsp60, wells of microtitre plates were coated with a recombinant human Hsp60 protein (Stress Gen, Victoria, British Columbia, Canada) (10 mg protein/well) and blocked with PBS containing 10% skimmed milk. The plates were incubated with 1:100 diluted serum for two hours at room temperature and washed with PBS containing 0.05% Tween 20, and bound human antibodies were detected as described above.

IMMUNOBLOTTING

Sonicated *H pylori* extracts, HGC-27 cells, and the recombinant human Hsp60 were dissolved in 1% sodium dodecyl sulphate containing 5% 2-mercaptoethanol and separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (10% gel). The separated proteins

Figure 2 Immunoblots of HGC-27 cells allowed to react with serum from various patient and control groups. Lysates of HGC-27 cells were fractionated on 10% polyacrylamide gels and then immunoblotted. (A) Lane A, The membrane strip was allowed to react with LK-2 mouse monoclonal antibody to human Hsp60. Lanes 1–7, the strips were allowed to react with serum from patients with mucosa associated lymphoid tissue (MALT) lymphoma. Bands with an approximate molecular mass of 60 kDa which correspond to Hsp60 are indicated by an arrow. (B) The membrane strip was allowed to react with serum from patients with non-ulcer dyspepsia (lanes 1–3), gastric ulcer (lanes 4–6), or duodenal ulcer (lanes 7 and 8), and from a healthy control subject (lane 9). Lane A, LK-2.

Figure 3 Immunoblots of recombinant human Hsp60 allowed to react with sera from patients with mucosa associated lymphoid tissue (MALT) lymphoma. Recombinant human Hsp60 proteins were fractionated on 10% polyacrylamide gels and then immunoblotted. Lane A, the membrane strip was allowed to react with LK-2 mouse monoclonal antibody to human Hsp60. Lanes 1–11, the strips were allowed to react with sera from patients with MALT lymphoma. Bands with approximate molecular mass 60 kDa, which correspond to Hsp60, are present in lanes 1–10 (arrow).

Table 1 Detection of human recombinant Hsp60 by immunoblotting

	No of patients	Hsp60 positivity
MALT lymphoma	11	10 (91)*
Gastric ulcer	20	3 (15)
Duodenal ulcer	20	3 (15)
Non-ulcer dyspepsia	20	4 (20)
Healthy control	10	0 (0)

Results in parentheses are percentages.

MALT, mucosa associated lymphoid tissue.

* $p < 0.01$ v the other patient groups and healthy controls (χ^2 test).

were electrophoretically transferred to polyvinylidene difluoride membrane (Millipore Japan, Tokyo, Japan). The membrane was cut into strips, and each strip was allowed to react with a patient's serum. After being washed with PBS containing 0.05% Tween 20, the strips were allowed to react with peroxidase conjugated rabbit anti-human IgG antibody, and the reaction products were detected using an ECL western blotting detection system (Amersham International, Little Chalfont, Bucks, UK).

Cross reactivity of human Hsp60 and components of *H. pylori* was examined by immunoblotting with mouse monoclonal anti-

body to Hsp60 (LK-2; Stress Gen). The membrane strips with proteins of *H. pylori* extracts, HGC-27 cells, and the human Hsp60 were prepared as described above and allowed to react with LK-2. After being washed, the strips were allowed to react with peroxidase conjugated swine anti-mouse IgG antibody (Dako) and developed using the chemiluminescence based detection kit as described above.

STATISTICAL ANALYSIS

Student's *t* test and the χ^2 test were used for statistical analyses.

Results

ANTIBODIES TO HUMAN GASTRIC CELLS

Figure 1 shows the distribution of antibody titres to HGC-27 human gastric epithelial cells in each group. Autoantibodies to HGC-27 cells were present in the serum of most patients with MALT lymphoma, and the antibody titres in these patients were significantly higher than those in the other patient groups and the healthy subjects ($p < 0.001$).

To define the target molecule(s) of the autoantibodies to HGC-27 cells, we performed immunoblotting using HGC-27 cells (fig 2). LK-2 anti-human Hsp60 mouse monoclonal antibody detected a band of molecular mass about 60 kDa, suggesting that HGC-27 cells constitutively express Hsp60. Serum from patients with MALT lymphoma detected various bands, and the band of molecular mass corresponding to Hsp60 was commonly observed in patients with MALT lymphoma (fig 2A). In contrast, serum from the other patient groups and the healthy control group rarely detected a band corresponding to Hsp60 (fig 2B).

Next, we tried to establish that the patients with MALT lymphoma had circulating antibodies to Hsp60, using immunoblotting and ELISA with the human recombinant Hsp60. On immunoblotting, 10 of 11 sera (91%) from patients with MALT lymphoma recognised a band of the recombinant human Hsp60 (fig 3), whereas sera from the other patient groups and the healthy control detected it significantly less often (0–20%, $p < 0.01$) (table 1). On ELISA, sera from patients with MALT lymphoma showed significantly higher antibody titres to the human Hsp60 than sera from the patients without MALT lymphoma and from the healthy control group ($p < 0.001$) (fig 4).

CROSS REACTIVITY OF LK2 ANTI-HUMAN HSP60

ANTIBODY TO *H. PYLORI*

LK-2 monoclonal antibody to the human Hsp60 recognised bands of 60 kDa in both *H. pylori* and HGC-27 cells, which probably correspond to *H. pylori* HspB^{12–14} and human Hsp60 respectively (fig 5).

Discussion

In this study, we found that serum antibody titres to HGC-27 human gastric epithelial cell were significantly elevated in *H. pylori* positive patients with MALT lymphoma when compared with titres in *H. pylori* positive patients who had other gastroduodenal diseases and in healthy subjects without *H. pylori* infection. Our

Figure 4 Serum antibody titres to recombinant human Hsp60 in patients with mucosa associated lymphoid tissue (MALT) lymphoma, gastric ulcer, duodenal ulcer, or non-ulcer dyspepsia, and control healthy subjects. Serum antibodies to human Hsp60 were measured using ELISA with plates coated with recombinant human Hsp60. * $p < 0.001$ (Student's *t* test).

Figure 5 Immunoblots with LK-2 mouse monoclonal antibody to human Hsp60. Recombinant human HSP60 (lane A), lysates of HGC-27 cells (lane B), and sonicates of *H. pylori* (lane C) were fractionated on 10% polyacrylamide gels and then immunoblotted. The membrane strip was allowed to react with LK-2 mouse monoclonal antibody to human Hsp60. Bands with approximate molecular mass 60 kDa (Hsp60 in lane A) are also present in lanes B and C.

immunoblot experiments showed that sera from patients with MALT lymphoma often detected a band with a molecular mass corresponding to that of Hsp60. Moreover elevated antibody titres to the recombinant human Hsp60 were shown by ELISA and immunoblotting. Thus Hsp60 is probably a major antigen recognised by the anti-HGC-27 antibodies present in serum of patients with MALT lymphoma.

The LK-2 anti-human Hsp60 antibody recognises the epitope encoded by amino acid residues 383 to 419 of the human Hsp60 protein.¹⁵ We found that this antibody also detected a band corresponding to bacterial HspB in *H. pylori* extracts, suggesting that a common epitope(s) is present in human Hsp60 and its bacterial homologue, HspB. Thus infection of *H. pylori* may induce antibodies against bacterial HspB which cross react with a host Hsp60 through the molecular mimicry of these proteins,¹² leading to the elevation of antibodies to HGC-27 cells in patients with MALT lymphoma. We have recently observed staining with LK-2 anti-Hsp60 antibody in follicular dendritic cells in germinal centres of gastric mucosa from patients with MALT lymphoma,¹⁶ suggesting that the antigen recognised by the anti-Hsp60 antibody which originates from either the bacteria or the host cell is presented to immune cells in this disease.

We found that the immune response to Hsp60 was closely associated with MALT lymphoma. Although the association of MALT lymphoma with *H. pylori* strains expressing the CagA protein has been reported,¹⁷ no specific differences in HspB protein amino acid sequences between bacterial strains from MALT lymphoma and those from other gastroduodenal diseases have been demonstrated. However, production of antibody to Hsp60 was less marked in other diseases with *H. pylori* infection. Undefined bacterial changes or host genetic backgrounds of immune responses to the bacterial infection may lead to enhanced immune responses to Hsp60 in patients with MALT lymphoma, but the reason for this specific response awaits clarification.

Induction of immune responses to host components may lead to tissue injury of an autoimmune nature. It is well known that gastric mucosal lesions of MALT lymphoma are often resistant to ordinary peptic ulcer treatment—that is, control of acid secretion. Immunological responses to *H. pylori* of both neoplastic B cells and non-neoplastic T cells have been reported in MALT lymphoma.¹⁸ In addition, gastric ulcers developed in severe combined immunodeficient mice transplanted with peripheral blood mononuclear cells from patients with MALT lymphoma when *H. pylori* was orally inoculated.¹⁹ These observations suggest that the development of gastric mucosal damage in patients with MALT lymphoma involves host immune responses to *H. pylori*. Hsp60 may be one of the target molecules.

In this study, a few *H. pylori* infected patients with gastric disease other than MALT lymphoma also had elevated IgG titres to HGC-27 cells and Hsp60. We are carefully following these patients to see whether they will develop gastric MALT lymphoma. In addition, the common association of anti-Hsp60 and anti-HGC-27 antibodies with MALT lymphoma suggests that measurement of these antibodies may provide a new diagnostic indicator for the disease.

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