Adherence and invasion of mouse-adapted *H pylori* in different epithelial cell lines

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Abstract

AIM: To assess the adhesion and invasion abilities of different mouse adapted *H pylori* strains in different cell lines *in vitro* and investigate their effects on the virulence factors cagA and vacA.

METHODS: The adherence and invasion abilities of different *H pylori* strains in different epithelial cell lines were examined by the gentamycin protection assay. The null mutants of cagA and vacA were processed by direct PCR mutation method. The morphologic changes of different cell lines after *H pylori* attachment were examined by microscopy.

RESULTS: The densities of adherence to and invasion into cells *in vitro* were different from those in the mouse infection experiments. 88-3887 strain could invade and adhere to cells stronger than SS1 and X47. All tested strains had better adhering and invasive abilities in SCG-7901 cell. CagA and vacA minus mutants had the same invasion and adherent abilities as their wild types. In all strains and cell lines tested, only AGS cell had the significant hummingbird phenotype after inoculation with the 88-3887 wild-type.

CONCLUSION: Both the host cells and the bacteria play important parts in the invasion and adhesion abilities of *H pylori*. CagA and VacA are not related to the ability of invasion and adhesion of *H pylori* in different cell lines *in vitro*.

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INTRODUCTION

Infection with the human gastric pathogen *H pylori* can develop into chronic gastritis, peptic ulcer and gastric cancer[1-4]. Adhesion to the gastric epithelium and the host responses take the crucial role in the pathogenesis of *H pylori* infections.

Adherence is considered to be associated with both colonization and virulence properties of *H pylori*. A mouse infection model has been used to investigate the pathogenesis of *H pylori*[5-8]. Results from Dr. Berg. DE and colleagues suggested that different *H pylori* strains had gradient colonization abilities in various mouse cell lines *in vivo* (unpublished data). Studies at cellular levels showed *H pylori* induced the secretion of pro-inflammatory cytokines, cell proliferation, and apoptosis of epithelial cells and the cell lines were used as models for the investigation of the interaction between the bacteria and hosts *in vitro*[9-12]. There were proofs that *H pylori* were not present in the gastric epithelial cells but in the mucus layer overlying the gastric tissue[1,13]. However, there were also a number of biopsy studies[14-16] and cell culture infection models[17-21] that provided increasing evidence for the intracellular survival of *H pylori*. Internalization into the host cell should be considered a major strategy to evade the host immune response of the bacteria. Amieva *et al*[22] proved that the intercellular *H pylori* may be released to repopulate the extracellular environment again. It is well accepted that *H pylori* infection is hard to be eradicated in some cases, and triple drug therapy often fails to eliminate *H pylori* in infected patients[23-25].

In this study, the adherence and invasion abilities of 3 mouse-adapted *H pylori* strains, which colonized the mice at different densities in different epithelial cells lines, were assessed by gentamycin adherence and invasiveness assay. Moreover, to investigate whether the virulence factors cagA and vacA could affect these abilities, mutants in these genes were established to examine their capacities to adhere to epithelial cells and compared to their wild-type counterparts.

MATERIALS AND METHODS

Bacteria and culture condition

The backgrounds of 3 mouse passed *H pylori* strains are shown in Table 1. All these mouse passed strains were

kindly offered by Dr. Berg DE, (Washington University, School of Medicine, St. Louis, MO 63110). The mouse inoculation experiments were done with C57BL/6J IL-12 KO mouse by Dr Berg DE's lab and the results from all these 4 strains were obtained. 88-3887 is a mouse adapted strain parentally from *H pylori* 26695. So far, there has been no reported data on the mouse colonization capacity of 88-3887 cagA::cam and SS1 vacA::cam.

Wild type *H pylori* strains of SS1, X47 and 88-3887 were grown in an *H pylori* selective medium (Columbia Agar Base supplemented with 5% sheep blood and 0.25 mg/mL vancomycin, 0.2 mg/mL amphotericin B, 0.2 mg/mL polymyxin B and 0.3 mg/mL trimethoprim) and incubated at 37℃ in a microaerobic atmosphere containing 5% O_2, 10% CO_2 and 85% N_2. CagA and vacA null mutants (88-3887 cagA::cam, SS1 vacA::cam) were grown in an *H pylori* selective medium with 20 μg/mL chloramphenicol and cultured under the same condition as wild type strains.

Generation of H pylori allelic replacement mutants

The null mutants for cagA::cam and vacA::cam were generated by direct PCR method as described previously[26,27]. Chloramphenicol resistance cassette (cat) was amplified using primers C1 and C2 designated from the pBlueScript Ⅱ SK plasmid. The alleles in which cagA and vacA were replaced by cat were generated by the PCR method. The PCR products containing those ΔcagA (cagA::cam) and ΔvacA (vacA::cam) alleles were used to transform the wild-type *H pylori* strains 88-3887 and SS1 with selection for Camr (20 μg/mL). The primers for 88-3887 ΔcagA (88-3887 ΔcagA::cam) were cagA-P1, cagA-P2, cagA-P3 and cagA-P4. Primers for SS1 ΔvacA (SS1 ΔvacA::cam) mutant were vacA-P1, vacA-P2, vacA-P3 and vacA-P4. The sequences of the primers are listed in Table 2. All mutations were confirmed by specific PCR using cat, vacA and cagA genes.

Preparation of cell line cultures

AGS cells (ATCC CRL 1739, human gastric adenocarcinoma epithelial cell line), SGC-7901 cells (human gastric cancer cell line from laboratory collection), MDCK cells (ATCC CCL-34, Marbin-Darby canine kidney epithelial cell line) were seeded to generate 2×10^5 cells in RPMI 1640 medium supplemented with 10% FCS (Gibco BRL, Eggenstein, Germany) per well in 24-well tissue culture plates. The plates were incubated at 37℃ in 5% CO_2 for 24 h. The culture medium was replaced with fresh RPMI1640 medium without FCS for 2 h before the inoculation of bacteria.

Adherence and invasiveness assay

The 24 h cultured bacteria were harvested from plates with phosphate-buffered saline (PBS) and washed by centrifugation at 5000 r/min for 5 min at 4℃ 3 times. The pellets were suspended in PBS to 1 OD600 (approximately 108 CFU/mL), and 0.1 mL of this suspension was inoculated into each duplicate host cell wells to achieve a multiplicity of infection (MOI) of 100. As a control, 0.1 mL of PBS was added to each host cell lines. Infection was carried out at 37℃ in 5% CO_2 for 5 h. After 5 h infection,

Table 1 Background of mouse passed *H pylori* strains

H pylori strains	CagA	Tissue tropism	CFU/g of mouse stomach tissue
SS1	+	Antrum	10^6
X47	-	Corpus	10^6
88-3887	+	[1]None	10^4

[1]Same distribution densities in antrum and corpus.

Table 2 Primers for null mutant of cagA and vacA of *H pylori*

Primer	Sequence (5'-3')
C1	GATATAGATTGAAAAGTGGAT
C2	TTATCAGTGCGACAAACTGGG
CagA-P1	CCCAAGCTGATCAGAGTGAG
CagA-P2	ATCCACTTTTCAATCTATATCGGCTTCGTTAGTCATTG TTTCTCC
CagA-P3	CCCAGTTTGTCGCACTGATAAGGTGGTTTCCAAAAAT CTTAAAGGATT
CagA-P4	GGTTGCACGCATTTTCCCTTAATC
VacA-P1	CTACGGTGTATGATGACGCTCA
VacA-P2	ATCCACTTTTCAATCTATATCCACAAAGGGTGCGAC TTTAGAC
VacA-P3	CCCAGTTTGTCGCACTGATAAGATCAATCAAGCTTG AATTTCA
VacA-P4	TTAGAAACTATACCTCATTCCTAAA

the monolayer was then washed 3 times with 1 mL RPMI1640 with 10% FCS medium and reincubated under the same conditions for another 2 h. Cell culture medium containing 25 mg/mL of gentamicin was added to each of the wells for the enumeration of intracellular bacteria (In preliminary experiments, 25 mg/mL of gentamicin could kill all *H pylori* isolates after a 2 h exposure and 0.1% saponin had no influence on *H pylori* culture compared with PBS). Following incubation, all monolayers (with or without gentamicin) were washed 5 times with RPMI 1640 and lysed with 0.1% saponin (Sigma cat#S-7900) in PBS. The suspensions were serial diluted. Both the intracellular bacteria and the total cell-associated bacteria were estimated by counting the number of CFU on *H pylori* selective plates. Results are expressed as the average from 3 independent experiments. Wild type *H pylori* strains and their mutants were inoculated into different cell lines respectively and mixed simultaneously. For the mix infection, the bacteria were counted on the normal selective medium and the selective medium with 20 μg/mL chloramphenicol separately.

Image capture

After the co-culture of bacteria and different cells for 5 h, the monolayer cells were washed by PBS and the images were obtained under the microscope (Olympus BX 51).

RESULTS

Invasion and adherence abilities of H pylori strains in different cell lines

SS1, X47 strains had higher capacities to colonize the mice than 88-3887 but the intensities of their invasion and adherence in the cell lines were quite different. 88-3887
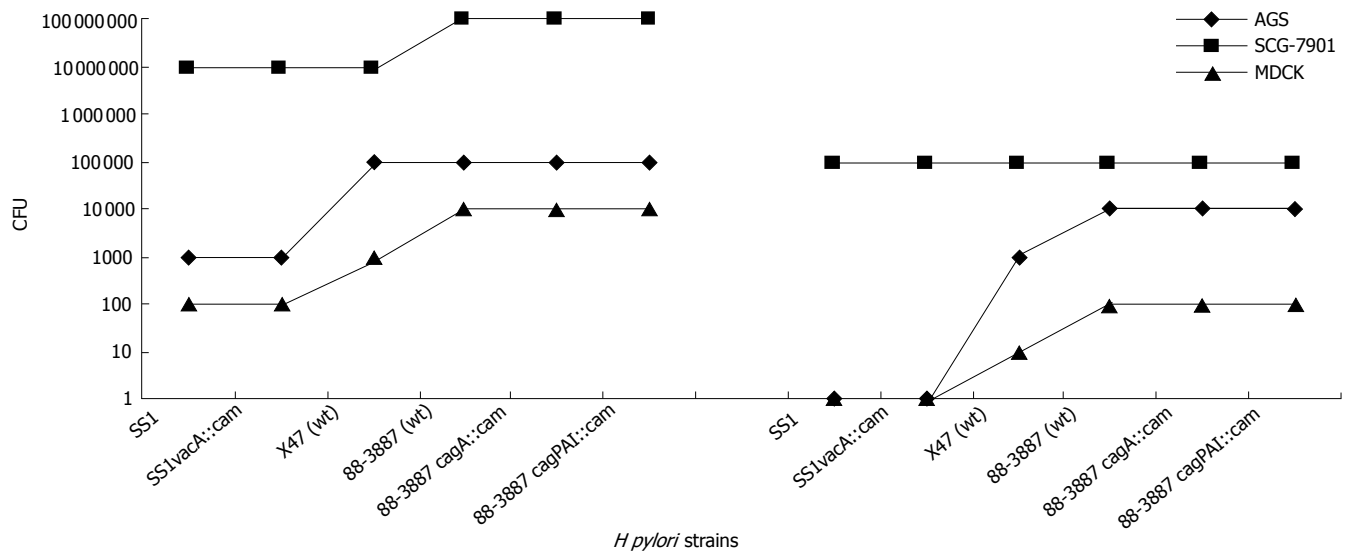
Figure 1 Levels of adherence and invasion of *H pylori* in different cell lines. Bacteria were added to AGS cell monolayer at an MOI of 100 for 5 h at 37℃ in a humidified atmosphere with 5% CO_2. Data were obtained by adherence and invasiveness assays in three independent experiments and are expressed as CFU per well of AGS cells. The left side 3 refrangible lines indicate the ability of the adherence in 3 different cell lines. The right sides indicate the invasiveness ability in the 3 cell lines. Values represent the mean CFU of viable bacteria recovered per well of a 24-well tissue culture tray.
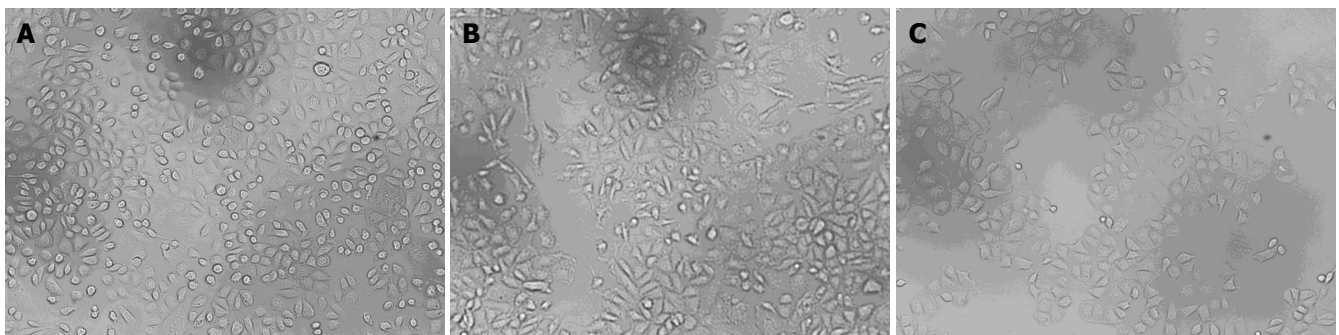

Figure 2 Images showing morphologic changes induced by *H pylori* attachment. After co-culture of the bacteria with different cells for 5 h, the monolayer AGS cells were washed with PBS and the images were captured by microscopic Olympus BX51. **A**: AGS cell; **B**: The hummingbird/scattering appearance of AGS after being attached with 88-3887 wild-type, 5 h; **C**: AGS with *H pylori* 88-3887 cagA minus mutant (88-3887 cagA::cam), 5 h.

could invade and adhere to the cells better than SS1 and X47. The intensity of SS1 was lowest among the three tested strains. Each of the three *H pylori* strains had the highest adherent and invasive ability in the SCG-7901 cell. 88-3887 ΔcagA::cam and SS1 ΔvacA::cam had the same invasiveness and adherence ability as their wild type counterparts, respectively. The effects of different bacteria in different cell lines are shown in Figure 1.

Morphologic changes of different cell lines after inoculation with H pylori

Cell morphology was examined under the microscope after 5 h co-culture. Fifty percent AGS cells became elongated like the hummingbird phenotype after attachment with 88-3887 wild type strain, while there were no such hummingbird like changes in AGS cell after inoculation with 88-3887 ΔcagA::cam. There were no significant hummingbird like phenotype in AGS after attachment with X47, SS1 and SS1vacA::cam strains. MDCK cells were scattered and slightly elongated after inoculation with 88-3887, 88-3887 ΔcagA::cam, SS1 and SS1 ΔvacA::

cam strains. After inoculation with X47 wild type strains, MDCK cells changed to round shape. The attachment of the 5 tested *H pylori* strains caused the SCG-7901 cells to become scattered, and there was no significant difference in phenotypic changes between different strains. The morphologic images are shown in Figure 2.

DISCUSSION

H pylori is one of the most prevalent and persistent infectious agents in humans[28-30]. Adhesion is considered to be a major process for *H pylori* to colonize the host tissue and cause diseases. Intimate attachment to the host cells could facilitate the bacterium's colonization, efficient delivery of effecter proteins such as cagA and vacA from the bacteria to the host cell and gain of nutrients from the environment. Bacteria with better adherence properties would colonize the host at higher densities and cause severe damage of the host[31,32]. Although *H pylori* have generally been considered as an extracellular pathogen, a number of *in vitro* infection experiments and biopsy

examinations have shown that it is capable of occasionally entering mammalian cells[33]. This penetration action may be one of the mechanisms for survival of the bacteria. In the present study, all tested H pylori strains adhered to SCG-7901, AGS and MDCK cells in a gradient way and the densities were 10^{7-8}, 10^{3-5} and 10^{2-4} CFU/mL, respectively. The intensities of the adhering ability in different cells in vitro were quite different from those in the mouse infection models in vivo and the H pylori adherence capacities in human cells were stronger than that in the canine kidney epithelial cells. According to this study, the invasive capacity was always consistent with the adhesion ability in all the tested cell lines, the higher the adhesion capacity, the better the invasion ability. Our results are consistent with previous evidence of the invasion ability in mammalian cells of H pylori[22,34] and it may imply the varied attachment mechanisms among different host species during H pylori infection. It also supports the view that both the host and bacterial factors mediate the adhesion and invasion of H pylori.

In the present study, the gentamicin protection assay was relatively simple, reproducible and measureable. Bacteria were determined after recovery from an additional 2 h incubation with gentamicin in the medium. The internalized bacteria were protected since the gentamicin could not penetrate the mammalian cell membranes. All the calculated data were repeated 3 times and each host cell had duplicate wells for each H pylori strain for validation.

CagA is present in some H pylori strains with enhanced virulence, and has been identified as an important risk factor for development of severe gastric diseases. H pylori strains are divided into two groups named type Ⅰ and type Ⅱ strains, based on whether they express cagA or not[35,36]. It was previously described that cagA was an effector protein of H pylori that was translocated via a type Ⅳ secretion system into gastric epithelial cells, interacted with different components in the host cell signal transduction pathways and the actin binding proteins, which ultimately affect the cytoskeletal organization[37-40]. This study showes no correlation between the presence of cagA with the ability of adhesion and invasion of H pylori. The only hummingbird response of AGS cell was induced by 88-3887 wild type strain but not the cagA minus mutant. This result is consistent with the previous conclusion that cagA plays a crucial role in the host cytoskeleton change. However, neither SS1 (cagA+) nor X47 (cagA-) could induce the same morphologic changes in the tested cells. Apart from AGS with the 88-3887 wild type, the other tested host cells attached with H pylori presented the under stress phenotypic changes such as cell scattering, elongation, and roundness in shape. These induced changes were not necessarily associated with cagA or vacA. It has been discovered that translocated cagA forms a physical complex with tyrosine phosphatase SHP-2, which plays an important role in the signal transduction pathway of the cell skeleton. According to the sequences constituting the SHP-2 binding site, cagA proteins can be sub-classified into East Asian and Western types, which have different binding and transforming activities[41,42]; while SS1 and 88-3887 all belong to the Western type. However, this could not explain why SS1

did not induce the same phenotypic change in AGS as 88-3887. SS1 also harbors cagA and could achieve high level mouse colonization. But this is controversial to the fact that the cag PAI in this strain is incomplete, as it lacks one open reading frame in the so-called 'left half' of the island[43,44]. On the other hand, we had no evidence to support that the gene defect SS1 strain caused different effects on AGS phenotypic changes from that of 88-3887 strain. Dana et al[45] reported that mouse adapted strains had a reduced capacity to induce inflammatory responses in AGS cells and suggests that such bacteria are more easily to colonize mice. The present results may indicate that the effector function of cagA in the host cell cytoskeletal rearrangement is pivotal and bacterium and host dependent. In different hosts, different H pylori strains invade via alternate receptor mechanisms, and various signal transduction pathways. The high degree genetic diversities of H pylori may be generated depending on the geographic origin or the ethnic origin of the host.

VacA cytotoxin is also considered to be an important virulence factor in H pylori, since it induces large cytoplasmic vacuoles in cultured mammalian cells. VacA is present in all H pylori strains and has been shown to be related to the colonization and intracellular survival of the bacteria[46,47]. This study shows that vacA has no effect on the adhesion and invasion abilities of H pylori. Our results are conflict with Terebiznik's data but consistent with Amieva et al[22]'s observation that the internalization of H pylori did not require vacA or cagA[48]. The phenotypic changes of the host cells attached with SS1 vacA minus mutant were the same as the wild type H pylori strains.

In summary, this study assesses the adherence and invasiveness abilities of different mouse adapted H pylori strains in different mammalian cell lines. The results imply that both the host-genetic and the microbial factors are involved in the development of the pathogenic infection outcome. VacA and cagA constitute important virulence factors of H pylori and both are delivered into the host cells depending on the intimate contact between bacteria and the gastric epithelial cells. This study suggests that neither vacA nor cagA is associated with the adhesion and invasion abilities of H pylori in different mammalian cell models. The phenotypic changes induced by H pylori are also host and bacterium dependent.

ACKNOWLEDGMENTS

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