Abstract. – OBJECTIVE: Helicobacter pylori (Hp) is a pathogen which causes gastric diseases from gastritis to ulcer, cancer, and MALT Lymphoma. The higher grade of virulence belongs to strains harbouring CagA oncoprotein. Hp cag Pathogenicity Island encoded Type IV Secretion System (T4SS) is supposed to form needle-like structures (pili) on Hp surface that injects cagA into the host cell cytoplasm at basolateral membrane level. Other structures such as membranous appendages have also been described on Hp surface membrane. These data were obtained from cultured cells. The aim of the present, preliminary, retrospective, study was to investigate the morphological expression of T4SS also in human gastric biopsies and revisit the morphological aspects of bacteria and host interaction.

MATERIALS AND METHODS: We reviewed our previous morphological findings on over 300 scanning electron micrographs of 109 gastric biopsic specimens.

RESULTS: 1) Needle-like structures (Pili) and membranous appendages have been found at the Hp gastric cell surface interface; 2) Hp polar flagella merge to apical mucous cell membrane; 3) some gastric cell microvilli became taller and projected towards bacteria; 4) in lining epithelium basal membrane holes in which bacteria penetrate are clearly visible.

CONCLUSIONS: Hp T4SS associated structures have also been observed in human biopsies. Pili could also take nutrients from gastric mucous cells (iron); the polar flagella could anchor Hp tightly to mucous cells; the tall microvilli projected to Hp could represent defence or affinity.

Key Words: Helicobacter pylori, Gastric mucosa, Cag secretion system, Electron microscopy.

Introduction

Helicobacter pylori (Hp) is a pathogen, which causes gastric mucosal damage ranging from gastritis to ulcer, adenocarcinoma or gastric MALT lymphoma, and extra-gastric diseases as well. The higher grade of virulence belongs to strains harbouring the Cag Pathogenicity Island (CagPAI); CagA positive strains increase the risk to develop atrophic gastritis and gastric cancer. However, in a recent review, the oncogenic pathogenicity of Hp has been revisited on the basis of current evidence: not only bacterial factors, but also host and environmental ones enhancing the severity, extent and duration of inflammation, increase the risk of gastric cancer.

Cag Pathogenicity Island encodes proteins forming a Type IV Secretion System (T4SS). Evidence derives from studies performed in cultured cells. Upon the contact between bacteria and host, T4SS forms needle-like structures on Hp surface, named pili. Helicobacter pylori secretes a protein, the serine protease HtrA, which cleaves the junctional complex proteins Occludin, Claudin-8, E-Cadherin; this is supposed to cause the para cellular transmigration of Hp, which reaches the basolateral cell membrane and binds to beta 1 integrin receptor. Tegtmeyer et al. reported that T4SS pili activation occurs at this level and induces the injection of the CagA oncoprotein into the cell cytoplasm.

Recently Chang et al. performed on cultured cells a study dealing with Hp Cag Type IV Secretion System. This system produces tube-like appendages when it gets in touch with epithelial cells; it is supposed to transfer effector molecules between microbes and from them into eukaryotic cells. The authors demonstrated by electron cryo-tomography the membranous structure of the tubes, which showed lateral ports.

To date, T4SS has been investigated only in cultured cells by morphological submicroscopic techniques. In the 80s we began to analyze extensively the human gastric mucosa in different pathological con-
ditions mainly by Scanning Electron Microscopy (SEM) applied to endoscopic biopsies. This submicroscopic morphological technique allows studying the three-dimensional features of lining mucous epithelium in extended areas. In 1983-84 we observed unidentifiable structures located in the intercellular spaces of surface mucous cell. We considered them contaminants or artefacts. After Marshall and Warren’s findings described in their paper of 1984, it became mandatory for us to review what we had mistakenly underestimated. Subsequently we applied a method further described in the text in order to remove the mucus covering the epithelial cells surface. We were able to observe the three-dimensional structure of Hp (still named *Campylobacter pylori*). We first analyzed the bacterium by scanning electron microscopy and also transmission electron microscopy when appropriate on human gastric biopsies and its relationship with gastric mucosal surface. At that time, we were not aware of the aforementioned T4SS structures.

Aim of this preliminary study was to investigate the possible presence of T4SS structures on the surface of Hp infecting human gastric mucosa, as well as to revisit the morphological aspects of the contact and interaction between bacteria and host mucous lining epithelium.

**Materials and Methods**

We conducted a retrospective study reviewing previous morphological findings documented by over 300 scanning electron micrographs obtained by the observation of 109 biotopic specimens, taken by endoscopy from 38 patients with upper GI tract symptoms. Specimen for SEM were rinsed in saline, fixed in 2.5% glutaraldehyde in 0.1 m phosphate buffer for 3 hours at 4°C, and then post-fixed in 1% osmium tetroxide for 1 hour at room temperature. After rinsing in the same buffer, the specimens were placed overnight in a solution of 1% HCL, then vortexed for 5 min, ethanol and critical point dried, and gold sputtered. Some specimens were re-processed for comparative transmission electron microscopy (TEM), by araldite embedding, ultrathin sectioning, uranyl acetate and lead citrate staining.

**Results**

**Different Relationship Patterns at Hp And Human Gastric Mucosa Interface**

The bacterial population has a patchy distribution on the gastric mucosal surface. No bacteria were found in intestinal metaplasia areas of the stomach: a clear-cut separation occurs between the two-different lining epithelial cells, even if close to each other (Figure 1). On the other hand Hp was found in areas of gastric metaplasia in duodenal bulb. Bacteria are mainly arranged at the intercellular spaces (Figures 1, 2), clumping and penetrating the apical junctional complex in almost all the mucosal infected samples (Figures 1, 2). Furthermore, Hp bacteria appear to have wrapped up the whole cell leading to its destruction (Figures 1, 2). Bacteria resting on or between microvillus tips or lying on the apical mucous cell membrane deprived of microvilli have also been observed (Figures 2, 5).

**New Morphological Findings**

1. Membranous appendages were located on the surface of bacterial body, along the lateral sides. Structures like little knobs were also observed on Hp outer membrane (Figure 3). Thinner needle-like structures (Pili) have been observed connecting Hp with the host mucous cell microvilli (Figure 3).
2. The bacterial outer membrane has irregular contours when observed by transmission electron microscopy, showing its glycocalix, a few non-rigid extroflexions (appendages) and a kind of small humps (Figure 4).
3. Polar flagella were observed adhering to apical mucous cell membrane (Figure 2).
4. Hp has been observed in contact with microvillus tips: the microvilli in this case were different from those of non-infected mucosa: they appeared quite taller, as if projected towards the bacteria; their tips showed a kind of pedestal (Figure 5).
5. Mucosal areas deprived of lining epithelium allowed us to observe the three-dimensional aspect of the basal membrane dividing the epithelium from the lamina propria. Holes, in which bacteria penetrate, are clearly visible (Figure 6).

All the above-mentioned patterns may coexist in the same mucosal specimen.

**Discussion**

In the present preliminary study we demonstrated first the presence of Hp T4SS structures in human biopsies of gastric mucosa. Pili are formed on contact with host cell. We observed their presence also when Hp touches the apical mucous cell membrane and not only at the cell basolateral level as it was reported in cultured cells where they are supposed to inject cagA into the cytoplasm. We suggest they contribute to the adhesion to cell and take available nutrients through internal channels. A research on pili production in different condition of iron availability shows that the number of pili increases in iron deficiency and decreases in the opposite condition. We described first by scanning electron microscopy the little knobs distributed throughout the Hp surface membrane. It is possible that the T4SS activated by the Hp-host contact produces the appendages from these knobs. Their role could be to transfer molecular proteins to cells.

The projection of taller microvilli towards Hp recalls what has been observed in cultured cel-
Helicobacter pylori at the gastric mucosa interface

ls where microvilli start to engulf Hp\(^5\). It can be supposed that it is a defence mechanism of the infected cell rather than an affinity of microvilli glycocalix towards surface glycoside residues of Hp membrane. We previously described the recognition of these residues on human gastric mucosa by lectins and that it was dependent on blood group\(^16\). In any case morphology does not support what previously hypothesized, that is Hp damages microvilli that flatten until they disappear\(^19\). Beta 1 integrin receptor for Hp-cagA is reported at the basolateral level of cells\(^20\). Multiple adhesins contribute to Hp pathogenicity\(^21\).

Surface mucous cells of normal gastric mucosa show different patterns of apical membrane as observed in SEM in the same biotic specimen, depending on the functional state of mucus granules secretion: microvilli, short and stubby, may cover the entire luminal surface or be localized at the periphery. In this case a progressive bulging of cell surface occurs leading to mucus granules secretion. Hp swimming through the thick surface mucus layer and reaching up to lining epithelium gets in touch with mucous cells in different functional conditions. It could directly adhere to apical membrane that is not defended by microvilli in the phase immediately preceding mucus secretion. This could result in surface cell disruption. Current data suggest that microvilli are damaged by bacteria and are lost; according to our findings it is not clear if microvilli deprivation in infected cells is due to the bacteria or if bacteria adhere also to naked cells in the functional state of mucus secretion. In normal mucosa, we observe bulging of luminal cell surface but not blebs; the latters, found in infected mucosa, result in apoptotic bodies.

The polar flagella could be the way for Hp, not only to swim through the mucus layer, but also to anchor tightly to mucous cells. Bacterial proteins such as adhesins have also been described allowing bacteria to stay firmly attached: beta 1 integrin receptor for Hp is located at the basolateral cell membrane. In fact Hp is not completely removed as expected even during the processing of specimen for submicroscopic techniques. We underline that the biotic specimens were washed even with HCL to remove outer mucus layer. Specific glycoside residues are supposed to exist on mucous cell membrane to which Hp binds; on the other hand, Hp glycocalix is a factor promoting cell recognition and cell-to-cell adhesion. This could depend on blood group of the host. Otherwise, how explain the exclusive binding of Hp to gastric cells. Hp has a clear tropism to creek into intercellular spaces and tight junctions where secretes the serine protease HtrA, which cleaves the apical junctional complex proteins disassembling the cytoskeleton, thus destroying the cell.

In this preliminary work, we could not investigate correctly on the prevalence of T4SS expression on cagA positive and negative strains. We demonstrated the presence of T4SS also in human biotic specimens.

Conclusions and Research Perspectives

These findings deserve a deepening to investigate Hp T4SS expression in a larger population group of infected patients both CagA positive and negative: is T4SS Secretion System only present in cagA strains, and if not what is the role of these structures.

The correlation between Hp colonization and adhesion to gastric mucous cells with the host blood group must be clarified. To date individuals with type A blood are supposed to have elevated susceptibility to Hp infection\(^22\).

The role of gastric cell microvilli during Hp infection is not yet clear. Do they represent a defense system or a factor of attraction for Hp?

Moreover, we first reported a scanning electron microscopy finding of bacteria drilling the basal membrane of mucous cell lining epithelium: what is their role, where are they going? Perhaps they could reach the mucosal capillaries, and then adhere to erythrocytes. A refractory iron...
deficiency anemia has been reported in blood A group-Hp positive patients, due to iron shift from host erythrocytes to bacteria.  

**Contributors**
The paper was designed by the first Author. All Authors contributed equally in writing the manuscript and in literature search.

**Conflict of Interest**
The Authors declare that they have no conflict of interest.

**References**


