HELICOBACTER SP. MICROORGANISMS DO NOT ALTER PROLIFERATIVE ACTIVITY OF GASTRIC EPITHELIAL CELLS IN NATURALLY INFECTED SWINE

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Received: September 22, 2010   Accepted: January 27, 2011

Abstract

In the presented study, an evaluation of influence of different Helicobacter species and gastritis on intensity of cellular proliferation in pyloric glands of pigs' stomach was performed. Samples of gastric antral mucosa obtained from 38 slaughtered pigs with known Helicobacter sp. and gastric inflammation statuses were stained with haematoxylin-eosin and immunohistochemically, for Ki67 antigen expression. Proliferative activity of epithelial cells was assessed by determination of: a ratio of proliferative zone length to gastric crypts length, an average percentage of cells showing Ki67 expression in proliferative zones of antral glands, and value of mitotic index in glands' proliferative zones. None of the comparisons revealed statistically significant differences between animal groups with or without gastric inflammation, as well as between groups with or without Helicobacter colonisation. Additionally, no statistically significant differences were found between the group of animals that were infected with Candidatus Helicobacter suis, and that with the stomach colonised by different species of Helicobacter microorganisms.

Key words: swine, stomach, Helicobacter, gastritis, epithelial cell proliferation.

The regular activity of the gastrointestinal tract is the result of functions of all cells that constitute this complicated body system. These cells include epithelium of superficial mucosa, highly specialised cells of gastric glands that synthesise and release gastric acid and lytic enzymes, cells of APUD system, and stem cells present in proliferative zones that differentiate into cells of other types. Efficient action of gastrointestinal system is extremely important in domestic animals such as swine, because it determines gaining high body mass by animal in the shortest period. All factors that can alter the balance between proliferation, differentiation, and death of epithelial cells of mucosal membrane can significantly affect an efficient action of the gastrointestinal system and thus economic results of pigs breeding.

The results of previous studies indicate that infection of gastric mucosa with Helicobacter organisms is common in pigs, and depending on the method of examination, the presence of these bacteria was detected in 9.4% to 62.5% of histological gastric samples and in 63.8% to 86.6% of animals examined by PCR (2, 3, 4, 7). The prevalence of the infection was very low before weaning, increased rapidly after weaning, and reached 90% in the adult animals (10). Gastric Helicobacter organisms are considered to be responsible for many pathological conditions of gastric mucosa, especially ulceration of pars oesophagea in swine (1, 2, 17), gastric and duodenal ulceration, chronic antral inflammation in humans, ferrets, wild felids, and finally for human gastric malignancies (adenocarcinoma, MALT type lymphoma). In recently published study including large group of animals (595 regularly slaughtered swine), the presence of Helicobacter DNA was revealed in 63% of swine with ulcerative gastric lesions and only in 24% of animals without gastric pathology (1). Additionally, experimental infection of pigs with Candidatus Helicobacter suis was associated with infiltration of lymphocytes and plasma cells in the antral mucosa, evolving to follicular gastritis; however, inflammatory response was present only in this area and no apparent inflammation of the fundic stomach region was detected in the infected animals (11).

The bacterial colonisation, as well as various pathological conditions in the organ can disturb the cell proliferation rate and, on the other hand, the disorders related to intensification of the proliferation can be responsible for the occurrence of clinical or subclinical disorders of the organ function. It was shown that in humans (6, 9, 13) and dogs (18), the colonisation with Helicobacter sp. can change the intensity of proliferation in proliferative zones of gastric glands. Additionally, in humans, the elimination of the infection leads to a retreat of disorders related to presence of the...
microorganisms including the normalisation of cell proliferative activity (6, 8).

Up to now, there have been no data evaluating the proliferation intensity of swine gastric epithelial cells, in a perspective of common infections with Helicobacter sp., as well as in relation to diagnosed pathologic conditions of the stomach. In the presented study an evaluation of influence of different Helicobacter species and gastritis on the intensity of cellular proliferation in pyloric glands of the stomach of swine was performed.

**Material and Methods**

The study material consisted of pyloric mucosa sections, sampled from 38 slaughtered fatteners. Specimens were fixed in 10% buffered formalin, embedded in paraffin and sliced into 4 µm sections. The sections were stained with haematoxylin-eosin and immunohistochemically for Ki67 antigen detection. Simultaneously, specimens for PCR were sampled. The PCR was targeted towards Helicobacter genus and Candidatus Helicobacter suis amplification, as described elsewhere (7). In order to determine the influence of different Helicobacter species on proliferative activity of gastric epithelial cells, depending on PCR results, the animals were divided into three groups: group I (non-HEL – control group) - animals without Helicobacter sp. colonisation; group II (non-CHS) – with Helicobacter sp. but without Candidatus Helicobacter suis; group III (CHS) – with Helicobacter sp. and Candidatus Helicobacter suis. Results of the proliferative activity of gastric cells were compared between these groups of animals.

Additionally, in order to evaluate the influence of inflammation of gastric mucosa on proliferative activity of gastric epithelial cells, another, independent of the former, division was made. Based on microscopic evaluation of gastric mucosa sections, the animals were divided into two groups: G0 – animals without observed inflammation in pyloric part of the stomach, and G+ - animals with observed inflammation in pyloric part of the stomach. Results of proliferative activity of gastric cells were compared between these groups of animals. Histopathologic criteria for the examination of gastric gastric mucosa with Helicobacter organism infection were described in details elsewhere (20).

Proliferative activity of the pyloric epithelial cells was assessed by immunohistochemical staining with antibody against Ki67 antigen, which is expressed in nuclei of dividing cells. Ki67 antigen expression was detected with monoclonal antibody: Mouse Anti-Human Clon MIB-1 (DAKO, kraj) in 1:100 dilution (according to the manufacturers advice). After the incubation, the sections were rinsed in the buffer and complex DAKO EnVision+TM, Peroxidase Mouse was added for a 30 min incubation period, in a humid chamber, at room temperature. Then the specimens were washed in TRIS buffer and diaminobenzidine was spotted under the microscopic control until the colour reaction was obtained. Proliferative activity of epithelial cells was assessed by following parameters:

- determination of gastric crypts length (GC; µm), length of the proliferative zones (PZ; µm), and ratio of proliferative zone length to gastric crypts length (PZ/GC; µm/µm) in pyloric glands (Fig. 1) worked out by computer vision analysis system Lucia v. 4.21.
- determination of an average percentage of cells showing Ki67 expression from all cells of pyloric gland proliferative zone (% Ki67; Fig. 1) in 10 vision fields of light microscope in magnification 400x.
- determination in HE stained slides the value of mitotic index (MI = average number of mitoses in 10 vision fields of light microscope in magnification 400x) in glands’ proliferative zones.

The results were analysed statistically with the use of statistic software PASW Statistics 18.0. The normality of the distribution was established by Kolmogorov-Smirnov test. The distribution for the data tested was normal, and for statistic calculations parametric tests were applied. Multiple comparisons between independent features (groups: CHS, non-CHS, non-HEL for MI, Z/C, and %Ki67) were calculated by one way ANOVA. For comparisons between two independent features (groups: G0 and G+ for MI, S/K, and Ki67) t-test was applied. The difference was regarded as significant when P≤0.05.

**Results**

In the presented study, genetic material specific for genus Helicobacter was detected in 24 out of 38 tested pigs. In 12 cases, the PCR results were also positive for Candidatus Helicobacter suis (CHS group), and in the other 12 samples the PCRs were negative for Candidatus Helicobacter suis (non-CHS group). Lack of the Helicobacter genetic material was noted in 14 samples, which were therefore considered as free from infection, and constituted a control group (non-HEL group). Inflammation of pyloric part of the stomach (group G+) was observed in 14 animals, while in 19 pigs no features of pyloric inflammation were observed (group G0). Positive reaction with MIB-1 antibody was observed in a proliferative zone of the pyloric glands (Fig. 2).

Mean value of the examined parameters, minimum, maximum, and standard deviation in particular groups of animals are listed in Tables 1 and 2. No statistically significant differences between animal groups with or without gastric inflammation, as well as between groups with or without Helicobacter colonisation were revealed. Additionally, no statistically significant differences were stated between the group of animals that were infected with Candidatus Helicobacter suis, and those with stomach colonised by different species of Helicobacter microorganisms.
Fig. 1. Pyloric glands of swine antral mucosa stained immunohistochemically with MIB-1 antibody (nucleuses expressing Ki67 antigen stained brown). Two segments of pyloric glands (gastric crypt - GC, proliferative zone - PZ) are schematically marked.

**Discussion**

In a perspective of observations that were done in dogs and humans infected with *Helicobacter* sp., the results of the presented study seem to be a little bit surprising, since it was noticed that the presence of stomach inflammation, as well as bacterial colonisation have no significant influence on the intensity of proliferative activity of pyloric gland cells in pigs. In humans infected with *Helicobacter pylori* and in dogs with GHLO colonisation, alterations concerning proliferative activity of cells in gastric gland proliferative zone, were observed (6, 8, 13). There are many factors that can affect the rate of cell division including: cytokines and other factors that are released by inflammatory cells (TGF-α, IL-1, IL-6, free radicals) or by infecting microorganisms (urease, other soluble substances), or connected with endocrine mechanisms regulated by antral G and D cells (14, 22). As previous study revealed, the presence of some *Helicobacter* species can alter the number of G and D cells, and especially the ratio of these cells in antral gastric glands in swine, and thus affect the proliferative activity of cells via hormonal mechanisms (19). However, so far there have been no studies concerning relation between gastric endocrine cell number or function, and proliferative activity of gastric glands in swine, and therefore the interpretation of the results of the presented study can be difficult.

It was shown that experimental infection of piglets with different *Helicobacter* species and other isolates can affect the gastric mucosa in various manners, and thus various pathogenic effects of different but related microorganisms is possible. For example, *Helicobacter pylori*-like bacteria, characterised as high pathogenic, may cause esophageal (9 out of 13 cases) and glandular part of the stomach (5 out 13 cases) ulceration, and provoke strong gastritis, including formation of lymphoid follicles (15). On the contrast, infection with *Helicobacter heilmannii* was accompanied by only limited inflammation and no ulcerative lesions were observed (15). It can be suspected that *Helicobacters* isolated from swine belong to different species of the genus, which can significantly differ in their pathogenicity, and bacteria harbouring the stomach of animals in the studied population could in a large part represent the species (or strains) of low virulence. In humans as well as in veterinary pathology, the fact of different pathogenicity of different *Helicobacter* species is well known (16). In the presented study, one of the studied groups consisted of animals infected with *Candidatus Helicobacter suis*, microorganism that is genetically very close and almost identical to *Helicobacter heilmannii* type 1, which, as it was demonstrated, has low pathogenicity (2, 5, 10, 15).

It is possible that the microorganisms that harbour pigs' stomach are not able to exert the visible impact on the proliferation of gastric mucosa cells due to lack of stimulating or inhibiting factors, or the intensity of colonisation is too small to exert the significant effect. The following interesting issue is that in the present study the *Helicobacter* infection did not cause any effect in terms of the association between bacterial colonisation and the number of endocrine cells in glands of pyloric part of the stomach, which was reported by other researchers (9). It is possible, that sole change in the number of particular cell type, or G to D cell ratio has no effect on the intensity of cell replication, and rather changes in endocrine cell activity are needed to alter the cell proliferation of gastric glands. On the opposite, bacterial infection and related disturbances in gastric homeostasis are not able to change the intensity of cells division in proliferative zones of the glands, but influence the differentiation of cells into one or another type, depending on change in the microenvironment.

Many factors, such as inflammation and activity of many produced multiple bioactive substances (interleukins, cytokines) could be responsible for the change in proliferation rate in pyloric gland proliferative zone of swine. However, the presented data did not prove such a possibility. When proliferation intensity was compared between animals with and without gastritis, no statistically significant differences were observed in the studied parameters. Results of earlier work did not determine whether the presence of *Helicobacter* organisms (no matter of species) was related to the presence or intensity of inflammatory cellular infiltrations in antral gastric mucosa (20).
Fig. 2. Expression of Ki67 antigen (brown stained nuclei) in pyloric glands of swine antral mucosa, in case of high (A) and low (B) mitotic activity. Immunohistochemical staining, MIB-1 antibody, 200x.

Table 1

| Parameters examined | Groups of animals |  |  |  |  |  |  |  |  |  |  |  |
|---------------------|-------------------|---|---|---|---|---|---|---|---|---|---|
| MI                  | Non-Hel group     | Mean value | Min | Max | SD | Mean value | Min | Max | SD | Mean value | Min | Max | SD |
| MI                  | CHS group         | 0.79 | 0.20 | 2.00 | 0.52 | 0.68 | 0.10 | 1.20 | 0.41 | 1.12 | 0.30 | 2.40 | 0.75 |
| PZ/GC               | Non-CHS group     | 0.46 | 0.34 | 0.75 | 0.17 | 0.39 | 0.24 | 0.50 | 0.08 | 0.42 | 0.22 | 0.55 | 0.10 |
| % Ki                |                   | 30.56 | 23.40 | 40.30 | 0.52 | 30.61 | 22.00 | 43.20 | 7.88 | 31.38 | 14.10 | 64.10 | 14.86 |

MI - mitotic index; PZ/GC - ratio of proliferative zone length to gastric crypts length; %Ki - percentage of cells showing Ki67 expression from all cells of pyloric glands proliferative zone; Min - minimum; Max - maximum; SD - standard deviation.

Table 2

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MI - mitotic index; PZ/GC - ratio of proliferative zone length to gastric crypts length; %Ki - percentage of cells showing Ki67 expression from all cells of pyloric glands proliferative zone; Min - minimum; Max - maximum; SD - standard deviation.
Inflammatory infiltration composition, which accompanies the presence of *Helicobacter* sp. is typical for chronic inflammation (domination of lymphocytes and plasmatic cells), with no features of active process (presence of neutrophils), which can determine the structural and functional lesions of gastric epithelial cells. Such a chronic active gastritis with undoubtful negative influence on the structure and function of gastric mucosa accompanies *Helicobacter pylori* infection in humans, and eradication of gastritis with accompanying a decrease in neutrophil number in gastric mucosa was associated with a decrease in proliferation rate (8).

The application of staining with MIB-1 antibody allows the detection of antigen Ki67 in nuclei of the cells in different stages of division cycle and is successfully used in the evaluation of cell proliferative activity in healthy, as well as pathological tissues. Although, in the presented study, MIB-1 antibody is designed for staining of human tissues, it seems that results of the swine tissue staining can be considered as reliable. In multiple papers from animal studies, including swine, mono- and polyclonal antibodies specific for human cell antigens are used, and results of this method are considered reliable (12, 21).

Mitotic index (MI) is an easy, cheap, and efficient test estimating proliferative activity of the various types of healthy and pathologic cells in humans and animals. Although previous study conducted on mucosa samples of canine stomach revealed poor usefulness of MI value as method of examination the proliferation rate, this method of examination was used in presented study to assess if it can be an useful parameter in estimation of proliferative activity of gastric epithelial cells in pigs (18). Unfortunately, the presented study did not reveal any significant differences in the proliferative activity between groups of examined animals, thus the usefulness of MI value could not be adequately estimated and compared to results of immunohistochemistry.

To make objective evaluation of the intensity of gastric epithelial cell proliferation in the presented study, two different parameters based on immunohistochemical staining of mucosal sections were applied. First, proportion of Ki67 positive cells to all cells present in gland proliferative zones was determined. This method was proposed by Fan et al. (6) and Havard et al. (9) and it seems to reflect intensity of cells division because the method shows the presence of cells in division cycle. Additionally, evaluated proportion of proliferative zone length to gastric pit length allows showing more objectively the actual lesions in proliferative zones, rather than only the measurement of sole length of proliferative zone, that can be easily influenced by the muscular mucosa constrictions.

Although gastric ulceration of swine stomach is localised in *pars oesophagea*, the choice to examine samples from gastric antral had many indications. First, according to formerly published data, bacterial colonisation, especially with *Candidatus Helicobacter suis*, was most common in the antral area of the swine stomach (7). Moreover, in the pyloric glands there is the highest number of endocrine cells, which control the function of the other gland cells and disorders in their proliferation can contribute to different pathological conditions of the stomach. Studies evaluating the effect of *Helicobacter pylori* infection on proliferative activity of stomach cells in humans were also conducted on samples of antral mucosa, because of significant influence of this area of the stomach on function and structure of other parts of the organ (6, 9). However, it seems that it would be advisable to conduct further investigation evaluating proliferation intensity in other parts of the swine stomach, especially in *pars oesophagea*, where pathologic lesions, including ulceration, are most commonly observed.

In summary, it has to be noted that the presence of *Helicobacter* organisms in swine antral mucosa do not alter proliferative activity of glandular cells, and there is no association between antral inflammation and epithelial proliferative activity.

References