MOLECULAR ALTERATIONS IN FIBROBLASTS EXPOSED TO \textit{HELICOBACTER PYLORI}: A MISSING LINK IN BACTERIAL INFLAMMATION PROGRESSING INTO GASTRIC CARCINOGENESIS?

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Major human pathogen \textit{Helicobacter pylori} (Hp) can colonize the gastric mucosa causing inflammation and being of potential risk for gastric cancer development but the contribution of fibroblasts to the pathogenesis of Hp in the stomach has been little studied. Normal stroma contains few fibroblasts, especially myofibroblasts, but their number rapidly increases in the reactive stroma surrounding inflammatory region and neoplastic tissue. We determined the effect of co-incubation of cultured rat gastric fibroblasts with alive Hp on the transdifferentiation of fibroblasts into myofibroblasts associated with Hp-induced inflammation and neoplasia. Gastric mucosal samples were harvested from 8-week-old Spraque-Dowley rats and cultured to obtain the sub-confluent fibroblasts. The isolated fibroblasts were infected with 1×10^6 of live Hp (ATCC 700824, cagA+, vacA+) per dish and incubated in humidified atmosphere for 3, 24 and 48 hours. At respective times, fibroblasts were harvested and the expression of mRNA for \(\alpha\)-smooth muscle actin (SMA), hypoxia inducible factor (HIF)-1\(\alpha\), collagen I, heat shock protein (HSP)-70, heme oxygenase (HO)-1, Bax and Ki67 transcripts was determined by RT-PCR with specific primers. Hp increased the transdifferentiation of fibroblasts into myofibroblasts as reflected by the time-dependent overexpression of mRNA for \(\alpha\)-SMA. The increased expression of HIF-1\(\alpha\) and collagen I was observed in fibroblasts co-cultured with Hp. The expression of HSP70 which was negligible in isolated fibroblasts incubated with vehicle (saline) showed time-dependent 2–3 fold increase in those incubated with Hp. The HO-1 mRNA was strongly expressed in rat gastric fibroblasts without or with the co-incubation with Hp. The mRNA for Bax was progressively downregulated within the time of incubation while no significant changes in expression of proliferation marker Ki67 were recorded. We conclude that Hp-induced transdifferentiation of fibroblasts into myofibroblasts involves an increased expression of the early carcinogenic marker HIF-1\(\alpha\), and inhibition of proapoptotic Bax expression, and 2) the overexpression of HSP70 and the unchanged expression HO-1 and Ki67 probably represent the enhanced protective activity of Hp-infected fibroblasts to maintain their own integrity under inflammatory action of this bacteria and its cytotoxins.

\textbf{Key words:} \textit{Helicobacter pylori}, myofibroblasts, transdifferentiation, \(\alpha\)-smooth muscle actin, heat shock protein-70, heme oxygenase-1, hypoxia inducible factor-1\(\alpha\)

INTRODUCTION

Surface epithelial cells of gastric mucosa are constantly subjected to injurious factors, such as alcohol, acid, base, hyperosmolar solution, stress, and the major human pathogen \textit{Helicobacter pylori} (Hp) (1-3). It is well known that Hp is a gram-negative, spiral shaped bacterium which colonizes the human stomach of about 50% of the world’s population. Hp colonizes mostly epithelium of the gastric lining and may also penetrate mucus, reaching pits of gastric glands (1). Although all Hp-infected subjects develop gastritis, approximately 80% of these individuals remain asymptomatic. Hp could cause the gastric duodenal ulcers, gastric cancer and mucosa-associated lymphoid tissues (MALT) lymphomas affecting about 15%, 1% and 0.1% of the population, respectively (4, 5). Accordingly, Hp was classified in 1994 by the World Health Organization (WHO) as a class I carcinogen.

Hp has created a series of adaptive mechanisms enabling survival and proliferation in acidic environment of stomach (1, 3). The most important mechanisms include: the ability to adhere to host epithelial cells, high enzymatic urease activity produced by Hp, which provides covering capacity for survival of bacteria through neutralising acidic content in the gastric lumen, as well as the presence of proton pump that removes an excess of hydrogen ion (1, 3). Majority of Hp infected patients develop inflammation of gastric mucosa by the increase of proinflammatory genes as well as direct modulation of cellular function, resulting in promotion of cell growth and the resistance to apoptosis (1, 4, 7). Inflammation triggered by Hp induces many mediators and cellular effectors that appear to be involved in carcinogenesis, such as a variety of cytokines, chemokines and growth factors as well as a prominent fall in the expression of tumor suppressor genes in those cells (4). The contribution of Hp-induced inflammation to the development of gastric cancer should be further elucidated (4).
Many cancers of digestive organs, some of which are caused by infectious agents, are known to arise on a background of chronic inflammation (8). This includes Hp-induced gastric cancer (8), in which epithelial cells of gastric mucosa are directly exposed to Hp and its cytotoxic products. Virulence factors encoded by the cytotoxin associated gene pathogenicity island (cagPAI) include CagA protein injected via the type IV secretion system (T4SS) into host target cells, thus contributing to the development of gastric cancer (8-11).

Recent studies suggest that also fibroblasts may alter the mRNA expression of structural and cell-cycle associated genes in the presence of Hp (12). It was reported that the stomach infection of Hp is associated with increased myofibroblasts abundance along with its increased proliferation and migration (13, 14).

Normal stroma contains few fibroblasts, especially myofibroblasts, but their number rapidly increase in reactive stroma surrounding inflammatory region and neoplastic tissue, particularly in the case of progression of stomach cancer. The origin of myofibroblasts remains controversial although fibroblasts are considered to be the main precursors of myofibroblasts. The transdifferentiation of fibroblasts into myofibroblasts is driven by cancer cell-derived cytokines i.e. TGF-B (15).

Myofibroblasts differ from fibroblasts with expression of alpha-smooth muscle actin (α-SMA), a 42kDa molecule, found as a predominant actin protein in aortic smooth muscle cells (16). The α-SMA plays a central role in wound contraction, being influenced by various growth factors and inflammatory cytokines (17). However, in oral wounds that heal without scar formation, another molecule FGR10P2 in fibroblasts is abundant (15). Indeed α-SMA positive myofibroblasts correlate with enhanced production and deposition of extracellular-matrix related proteins like collagen type I involved in the process of fibrosis (15, 18). Myofibroblasts contribute to various steps of the cancer progression including the increased cell invasion and the suppression of apoptosis (19, 20) as well as the stimulation of stromal cells with increased production of growth factors such as HGF that in turn, can affect epithelial cells and enhance various growth factors (20). The changes in the stroma may drive invasion of cancer cells and metastasis (20, 21).

In the light of recent studies, heat shock proteins (HSP) are recognized as important family of chaperon proteins involved in cell integrity and defense as well as agents participating in stress response associated with gastric infection of Hp (22). Among HSP family, in particular heat shock protein-70 (HSP70) seems to be responsible for maintenance of cell integrity under normal cell growth and at the pathophysiological condition such as colonization of gastric mucosa by Hp (22).

Recently HSP32 (heme oxygenase-1) has been shown to play a significant role in the mechanism of mucosal integrity of upper GI tract (23, 24). However, the role of heme oxygenase-1 (HO-1) in the process of cancer development may depend on the type, location and the degree of advancement of cancer. The upregulation of HO-1 gene exhibits a cytoprotective activity protecting healthy cells against their transformation into the cancer cells. On the other hand, HO-1 through its antiapoptotic and proangiogenic actions has been shown to facilitate the increase in cancer and its invasion. HO-1 may then act as a tumor promoter in stromal cells but as a tumor suppressor in cancer cells (30, 31).

This study, was designed to determine the effect of Hp on the rat gastric fibroblasts in vitro and to examine the influence of Hp on markers of transition of fibroblasts into myofibroblasts and extracellular matrix proteins, α-SMA and collagen I. We also determined the effect of incubation of fibroblasts with Hp to check its influence on fibroblast proliferation by assessing Ki67, a marker of cell proliferation. An attempt was made to examine the effect of Hp on induction of HSP70 in cultured rat gastric fibroblasts and expression of proapoptotic protein Bax.

**MATERIALS AND METHODS**

**Cell isolation technique and fibroblast infection with Helicobacter pylori**

Gastric samples were harvested from 8-week-old Sprague-Dowley rats and extensively washed with sterile phosphate-buffered saline (PBS) to remove contaminating debris. Primary fibroblast culture was established by mincing gastric biopsy into 1–2 mm² pieces with scissors and placing it in tissue culture flasks under sterile conditions. Growth medium RPMI-10 containing 10% fetal bovine serum and antibiotics were added and gently mixed with minced tissue. The flasks were maintained in a humidified atmosphere of 5% CO₂ at 37°C, and the medium was changed every 2 days. When the cells grew up to 80% of confluence and they were passaged using standard trypsinization techniques to establish a secondary cell culture as reported before (32, 33).

**Helicobacter pylori culture**

Stock cultures were maintained at ~70°C in Brucella Broth (Becton Dickinson, Franklin Lakes, USA) supplemented with 10% fetal bovine serum and 10% glycerol. The Hp strain expressing CagA and Vac cytotoxins (ATCC 700824, cagA+ vacA+) bacteria was purchased from American Type Culture Collection, and grown on Columbia Agar supplemented with 5% fresh horse blood (BioMerieux, Marcy l’Etoile, France). The culture plates were incubated under microaerophilic conditions at 37°C for 3–5 days. Before the coincubation with fibroblasts, Hp strain was suspended in sterile PBS and transferred to the dishes containing fibroblasts.

The sub-confluent fibroblasts were infected with 1x10⁶ of live Hp per dish and incubated in humidified atmosphere for 3, 24 and 48 hours. After incubation period, the total cellular RNA was isolated according to procedure reported before (34).

Expression of α-smooth muscle actin, collagen I, heat shock protein-70, heme oxygenase-1, Bax, Ki67 and heme oxygenase inducible factor-α transcripts in the rat gastric fibroblasts determined by real-time PCR

Expression of α-SMA, collagen I, HSP70, HO-1, Bax, Ki67 and HIF-1α transcripts in the rat gastric fibroblasts was
Table 1. Rat oligonucleotide primers for detection of mRNA by RT-PCR, annealing temperature and size of PCR products employed in the experimental protocol.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
<th>Size of PCR product</th>
</tr>
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<tbody>
<tr>
<td>α-SMA</td>
<td>Forward 5'- CAT CAG GCA GTT CGT AGC TC-3', Reverse 5'- CTA GAG TAG TGA ACA TCA TGA TC-3'</td>
<td>60°C</td>
<td>525 bp</td>
</tr>
<tr>
<td>HSP70</td>
<td>Forward 5'- CAA GAA TGC GCT CGA GTC CTA-3', Reverse 5'- GCA GAT GAC CTC CTG GCA CTT-3'</td>
<td>60°C</td>
<td>124 bp</td>
</tr>
<tr>
<td>Collagen</td>
<td>Forward 5'- GCC AAC AGT CGA TGC ACC-3', Reverse 5'- AGG GCC AAT GTC CAT TCC G-3'</td>
<td>60°C</td>
<td>177 bp</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Forward 5'- TCT GGA CTC CCT CTG CTG-3', Reverse 5'- GCT GCC CTT CTG ACT CTG-3'</td>
<td>61°C</td>
<td>510 bp</td>
</tr>
<tr>
<td>Bax</td>
<td>Forward 5'- CTG CCA ACC CAC CCT GGT CT Reverse 5'- TGG CAG CTC ACA TGT TTT CTT-3'</td>
<td>55°C</td>
<td>195 bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward 5'- TTG TAA CCA ACT GGG ACG ATA TGG-3', Reverse 5'- GAT CTT GAT CTC CCT CAT GGT GCT AGG-3'</td>
<td>54°C</td>
<td>764 bp</td>
</tr>
<tr>
<td>HO-1</td>
<td>Forward 5'- CTT GCA GAG AGA AGG CTA CAT GA-3', Reverse 5'- AGA GTC CCT CAC AGA CAG GT TT-3'</td>
<td>60°C</td>
<td>250 bp</td>
</tr>
<tr>
<td>Ki67</td>
<td>Forward 5'- AAC CAG GAC TGT GTG CTC TGT AA-3', Reverse 5'- CTC TTT TGG CTT CCA TTT CTTC-3'</td>
<td>60°C</td>
<td>209 bp</td>
</tr>
</tbody>
</table>

determined by RT-PCR, using specific primers (Table 1). Briefly, after the subsequent time of Hp incubation with fibroblasts, the cells were harvested and total cellular RNA was isolated according to Chomczynski and Sacchi method (34) using Trizol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. First strand cDNA was synthesized from total cellular RNA (2 μg) using Reverse Transcription System (Promega, Madison, USA). The PCR was carried out in an automatic DNA thermal cycler, using 1 μg cDNA and Promega PCR reagents. For amplification of α-SMA, collagen I, HSP70, HO-1, Bax, Ki67 and HIF-1α cDNA, gene-specific primers were used (SIGMA-Aldrich St. Louis, USA) (Table 1). Amplification of control rat β-actin was performed on the same samples to verify the RNA integrity. PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 μg/ml ethidium bromide and then visualized under UV light. Location of predicted PCR product was confirmed by using 0’Gene Ruler 50 bp DNA ladder (Fermentas, Life Sciences, San Francisco, USA) as a standard marker.

Statistical analysis

Statistical analysis of the data were performed with the use of Excel. Results are expressed as means ±S.E.M. from 6 samples per each group. Statistical significance of difference was determined using analysis of variance one-way ANOVA test. Further statistical analysis for post hoc comparisons was carried out with Scheffe test. Differences were considered as statistically significant at p<0.05.

RESULTS

Primary and secondary rat fibroblast culture

After harvesting gastric biopsy samples the primary fibroblast culture was established as shown in Fig. 1A. To confirm purity of fibroblasts in cell culture and to avoid contamination with epithelial cells, the primary culture was passaged three times and cultured in RPMI-1640 medium (Fig. 1B).

Differentialiation of fibroblasts exposed to Helicobacter pylori

Differentialiation of fibroblasts into myofibroblasts was assessed by determination of expression of myofibroblast marker α-SMA and by assessing changes in collagen I production. Using specific primers for α-SMA, collagen I and β-actin as an internal standard, single bands for each of cDNAs at the expected size were observed. The ratio of α-SMA and collagen I to β-actin for intact secondary fibroblast culture (control) and for 3, 24 and 48 hours of incubation with Hp are shown in Figs. 2A and 3A. Densitometric analysis of mRNA expression in control (not exposed to Hp) cells showed moderate level of α-SMA and relatively low level of collagen I and these effects were not significantly influenced by coincubation of fibroblasts with Hp at 3 h and 24 h, respectively (Figs. 2B and 3B). In contrast, ratio of mRNA expression (α-SMA and β-actin) level revealed that the expression of α-SMA mRNA was significantly upregulated at 48 h of co-culture with Hp. The parallel increase in expression of collagen I mRNA was recorded in fibroblasts co-cultured with Hp at 48 h following bacteria application (Figs. 2B and 3B).

Expression of heat shock protein-70 and heme oxygenase-1 mRNA, the apoptosis related gene Bax and proliferation marker Ki67 in cultured fibroblasts with or without co-incubation with Helicobacter pylori

The mRNA for stress and pro-apoptotic proteins were assessed in culture of gastric fibroblasts. As shown in Figs. 4A and 4B, the incubation of cagA and vacA positive Hp strain with gastric fibroblasts upregulated in a time-dependent manner the expression of HSP70 mRNA in these cells. This significant increase in HSP70 mRNA expression was observed already after 24 hours and subsequently noted at 48 h after exposure of cell culture to this bacteria. In contrast, the cagA and vacA positive Hp strain had no significant influence on the expression of HO-1 mRNA level in fibroblasts in any of the selected times of the incubation (from 3 h to 48 h) (Figs. 5A and 5B).

Next we examined the expression of mRNA for pro-apoptotic Bax and the expression of mRNA for proliferation marker Ki67 in fibroblasts exposed to Hp strain expressing CagA and VacA...
cytotoxins. As shown in Figs. 6A and 6B the expression of Bax mRNA was significantly decreased already after 3 hours of co-incubation with this bacteria and this significant inhibition persisted throughout 24 h and 48 h upon start of incubation. At 48 h of incubation a significant inhibition of the signal for Bax mRNA was observed below the value of Bax mRNA recorded at 3 h and 24 h. The signal of mRNA for Ki67 was also unchanged at 3 h of incubation of fibroblasts with Hp expressing CagA and VacA cytotoxins compared to vehicle control (Figs. 7A and 7B). At 24 h of fibroblasts incubation with this bacteria, the signal for Ki67 in fibroblasts failed to show significant difference compared with that recorded at 3 h of this incubation. Also proliferation at 48 h of coin incubation with Hp remained unchanged as it was estimated by proliferation marker Ki67 level of mRNA (Figs. 7A and 7B).

**Determination of hypoxia inducible factor-1α mRNA level in fibroblasts co-incubated with Helicobacter pylori**

The signal of mRNA for HIF-1α was not significantly altered at 3 h of incubation of fibroblasts with alive Hp CagA+, VacA+ (ATTC 700824) and the ratio of α-SMA over β-actin (panel B) at respective times of co-incubation with this bacteria. Results are mean ±S.E.M. of 6 determinations. Asterisk indicates a significant (p<0.05) change as compared to the value obtained at 3 h and 24 h of incubation with Hp.
significantly increased (p<0.05) over that measured at 3 h. This significant upregulation of HIF-1α gene had persisted at 48 h upon the incubation with this bacteria and the ratio of HIF-1α over β-actin mRNA reached significantly higher value over that determined at 24 h of incubation. Increase in HIF-1α mRNA expression was highly significant after 48 hours of fibroblast co-culture with Hp (Figs. 8A and 8B) parallel with the increase in expression of fibroblasts differentiation marker α-SMA (Fig. 1B).
DISCUSSION

The involvement of *Hp* in the pathogenesis of a variety of gastric disorders, including peptic ulcers, MALT lymphoma and gastric cancer is well documented, however, the importance of other non-epithelial cellular compartments in *Hp* pathology of the gut has been little studied. Impaired balance between aggressive bacterial and host defensive factors plays an
essential role in the different clinical outcomes of *Hp* infection in the host. As the stromal microenvironment is a complex structure composed of an extracellular matrix (ECM), activated fibroblasts and myofibroblasts, inflammatory cells, blood and lymphatic vessels, there is convincing evidence that any change in that system, can influence the normal architecture of epithelial cells and alter their functions by this non-epithelial component.

Fig. 7. RT-PCR analysis of mRNA expression for Ki67 (panel A) in rat gastric fibroblast at 3, 24 and 48 h of co-incubation with *Hp* (ATCC 700824) and the ratio of Ki67 over β-actin (panel B) at respective times of co-incubation of these cells with *Hp*. Results are mean ±S.E.M. of 6 determinations.

Fig. 8. RT-PCR analysis of mRNA expression for HIF-1α (panel A) in rat gastric fibroblast at 3, 24 and 48 h of co-incubation with *Hp* (ATCC 700824) and the ratio of HIF-1α over β-actin (panel A) at respective times of co-incubation of these cells with *Hp* (panel B). Asterisk indicates a significant change (p<0.05) compared to the value obtained at 3 h of incubation with *Hp*. Results are mean ±S.E.M. of 6 determinations. Asterisk indicates a significant change (p<0.05) as compared to the value obtained in control cells.
Therefore, the aim of our present study was to determine, whether gastric fibroblasts may represent cellular target of \textit{Hp} affecting process of proliferation, apoptosis and cell defense system. We assumed that the increased differentiation into myofibroblasts, collagen production and changes in proliferation and apoptosis rates can be implicated in the mechanism of interaction between fibroblasts and \textit{Hp}. Our data presented in this work indicate that \textit{Hp} increased differentiation of fibroblasts into myofibroblasts because the expression of \(\alpha\)-SMA was enhanced by this bacteria. This also indicates that stroma cells could be affected by \textit{Hp} suggesting that fibroblasts could act as the one of the major source of myofibroblasts.

Moreover, we found that the enhanced production of \(\alpha\)-SMA correlated with the expression of mRNA for collagen type I. That is in agreement with existing evidence showing that myofibroblasts positive for \(\alpha\)-SMA correlate with enhanced production and deposition of extracellular matrix related proteins including collagen type I - an event associated with fibrosis (15, 18, 19, 35, 36). Moreover, the presence of abundant myofibroblasts in gastric carcinoid tumours has been postulated (20).

Interactions between the neoplastic and non-neoplastic cells and extracellular matrix have been proposed to stimulate the extensive desmoplastic reaction (19, 35). At the molecular level, stromal production is promoted by the activation of multiple cancer cell-derived signaling pathways such as transforming growth factor \(\beta\) (TGF-\(\beta\)), hepatocyte growth factor (HGF/Met), fibroblast growth factors (FGFs), insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) acting via autocrine and paracrine mechanisms (19, 35). These receptor-mediated signaling cascades activated by growth factors lead to secretion of structural matrix components including proteoglycans, collagens and fibronectin as well as catalytically active enzymes such as proteinases (35).

Generally it is accepted, that when the epithelium changes, the stroma inevitably follows. In cancer, changes in the stroma drive invasion and metastasis, the hallmarks of malignancy. As it was mentioned before stromal changes at the invasion front include the appearance of myofibroblasts that take a part in various aspects of the cancer progression increased cell invasion, and the suppression of apoptosis (19, 35). The stimulation of stromal cells enhances the production of growth factors such as HGF in turn acting on epithelial cells (35). For instance, \(\alpha\)-SMA next to PDGFB-R is in fact regarded as CAF’s (cancer-associated fibroblasts) marker (36). According to literature the transdifferentiation of fibroblasts into myofibroblasts is mediated by a cancer cell-derived cytokines such as TGF-\(\beta\) (15). Here we show, that the presence of \textit{Hp} and direct contact of \textit{Hp} with fibroblasts itself can induce this process directly and it is capable of influencing the stroma architecture, without prior interactions with epithelial cells.

The concept suggesting that the microenvironment is crucial for the maintenance of cellular functions and tissue integrity indicates that a neoplasia-induced change in the stroma may contribute to cancer invasion (37-39). Experimental animal models have demonstrated that cancer invasion is stimulated by the wound-healing stroma (37-39). Therefore, in both wound healing and tumorgenesis, the transition of fibroblasts to myofibroblasts is an example of the stromal alteration. The casual role of myofibroblasts in the transition from the noninvasive towards the invasive phenotype is supported by the evidence that the appearance of myofibroblasts precedes the invasive stage of cancer (37-39). Experimental and clinical observations seem to suggest that myofibroblasts may be proinvasive and capable of producing proinvasive signals (37-39).

The evidence based scientific observations suggest that growth factors such as TGF-\(\beta\)I implicated in wound healing (38, 39) may also play a role in changing of the stromal host compartment in cancer. TGF-\(\beta\) is known to upregulate \(\alpha\)-SMA actin expression in fibrocytes and transdifferentiate them into myofibroblasts in many systems (15, 21, 37-39).

We also observed the enhancement in expression of mRNA for TGF-\(\beta\) (data not shown). But as given literature evidence, TGF-\(\beta\) is secreted as a latent complex that requires an activation that is regulated environmentally, either proteolytically or nonproteolytically. Host cells such myofibroblasts may play important role in turning TGF-\(\beta\) into biologically active, since they are considered as major suppliers of proteinases and thrombospondin (41). Fibroblast to myofibroblast transdifferentiation may be responsible for an activation of TGF-\(\beta\) in tumours (38-41).

The increase in myofibroblasts in colon cancer could result from transdifferentiation of resident interstitial fibroblasts into myofibroblasts or a decreased rate of apoptosis (38, 41). Our results suggest both transdifferentiation and decreased apoptosis in \textit{Hp} infected fibroblasts.

The quantity of ECM proteins, provide structural and functional scaffolding for cancer cells i.e. cytосkeletal organization, ectopic survival and differentiation (41) as well as shielding cancer cells from the immune response (41, 42). The collagen type I secretion downregulates E-cadherin gene expression and stabilizes epithelial to myofibroblast transdifferentiation (38, 41, 42). Since myofibroblasts are potentially invasive, they may invade collagen I so they may be able to migrate, invade and stimulate cancer cells and vice versa.

We found the enhanced upregulation of collagen I which is in agreement with the studies suggesting that CagA-positive strain of \textit{Hp} plays an important role in tissue remodeling, angiogenesis, cancer invasion and metastasis (43, 45). Moreover, studies in whole blood cultures revealed that \textit{Hp} strain expressing CagA cytotoxin exhibited immunomodulatory effect on expression of CD25 and CD58 receptors on lymphocytes and activation of cytokines such as IL-10 capable of inhibit the proinflammatory activity of monocytes and T lymphocytes (45). We propose, that some of these effects may be mediated by myofibroblasts because after eradication of \textit{Hp} inflammatory cell infiltration is reduced, but the advanced intestinal metastasization mucosa itself is usually not improved (46).

The accumulation and activation of HIF-1\(\alpha\) is a very challenging field of study since the responses are cell type specific and, therefore, may vary between cells of different origin. There is an increasing body of evidence that HIF-1\(\alpha\) plays an important role in various infections (26, 30, 43, 44), however, the mechanisms of HIF-1\(\alpha\) activation by \textit{Hp} are not well established. The study of Bhattacharyya et al. (27) have indicated that \textit{Hp}-mediated APE1 induction enhanced HIF-1\(\alpha\) expression and, along with p300, upregulated HIF-1\(\alpha\) transcriptional activity in the gastric epithelium. A cell line study has shown, that the reactive oxygen species produced by \textit{Hp}, stabilise HIF-1\(\alpha\), leading to its increased expression (28). The similar effect has been observed by Ding and coworkers (47).

The \textit{Hp} expressing CagA cytotoxin and also CagA by itself can induce ROS production \textit{via} NADPH oxidase-dependent pathway (48) causing DNA damage, thus contributing to apoptosis in early stages of gastric carcinogenesis. However, this was not the case with respect to apoptosis in our study because Bax expression was downregulated in fibroblasts incubated with \textit{Hp}. In culture of MCF7 cancer cells and fibroblasts, the enhanced ROS production and oxidative stress in adjacent fibroblasts as well as the activation of HIF-1\(\alpha\)- and NFκB driven gene transcription were observed. In their study (49), ROS were responsible for activation of myofibroblast markers and extracellular matrix proteins associated with activated fibroblasts. Therefore, we attempted to check if the same effect appears in our system, especially that, according to
existing evidence, many of the proteins induced by activated HIF-1α in fibroblasts are highly expressed in the tumor stroma of human breast cancer patients being particularly responsible for tumor recurrence or metastasis (49, 50). HIF-1α acts as a tumor promoter in stromal cells but as a tumor suppressor in cancer cells.

Tumour hypoxia is now recognised as a key factor driving the development of malignancy, and the master regulatory protein in the response of cells to change in oxygen levels via HIF-1α (49, 50). We postulate here that HIF-1α could represent one of the prerequisites to development of gastric cancer development in Hp infected mucosa. This is supported by the fact that HIF-1α expression was upregulated in fibroblast co-incubated with Hp.

As there is increasing evidence that apoptosis plays an important role in the pathogenesis of a variety of infectious diseases, we decided to check whether incubation of gastric fibroblasts with Hp influences the expression of HSP70, apoptosis related proteins Bax and expression of proliferation marker Ki67. In our culture model system, the cagA and vacA positive Hp strain upregulated in a time-dependent manner the expression of HSP70 mRNA in fibroblasts. This increase in HSP70 mRNA expression was observed already after 24 hours and subsequently noted at 48 h after exposure of cell culture to Hp. The expression of Bax mRNA significantly decreased in fibroblasts already after 3 hours of their co-incubation with bacteria. This finding indicates that Hp inhibits apoptosis of fibroblasts in vitro.

Previous studies revealed that epithelial cells co-incubation with Hp induces apoptosis and causes a fall in expression of HSP70 (22). This excessive apoptosis resulted in tissue damage. Targosz et al. (22) found that cagA, vacA positive Hp strains enhanced apoptosis in rat gastric cancer epithelial cells, as evidenced by an increase in expression of pro-apoptotic Bax and decrease in expression for anti-apoptotic Bcl-2. At present, the mechanism by which Hp induces apoptosis in gastric cancer cells and interacts with HSP70 expression remains unknown.

The major finding of their study (22) was that Hp can directly attenuate the expression of HSP70 in MKN7 cells which could accompany the rise in apoptosis. It has been postulated that inactivation of HSP90 and HSP70 leads to loss of invasion in a variety of cancer cell types (51). Thus, we conclude that the influence of Hp on HSP70 may be cell type specific. In the case of epithelial cells, Hp evoked an increase in apoptosis, while in the case of fibroblasts this bug evidently promoted their differentiation with sustained rate of proliferation.

It has been demonstrated that HO-1 mRNA and protein levels are strongly inducible in fibroblasts following i.e. oxidative stress (UV radiation and hydrogen peroxide) (52, 53). HO-1 activation exhibits a cytoprotective effect due to the anti-inflammatory, anti-apoptotic activity, and preserved control in regulation of proliferative activity (25, 26). In our experimental model the HO-1 mRNA was also strongly expressed, but we observed no alteration in HO-1 expression level following co-incubation with Hp. Lakkisto et al. (53) showed that, HO-1 decreased accumulation and proliferation of fibroblasts, and down-regulated procollagen type I expression in the infarct area of heart failure model in rats. Lack of major alteration in HO-1 expression under our experimental settings is in agreement with the studies pointing to antifibrogenic properties of HO-1. According to their accumulated evidence (53) the cardioprotective effects of HO-1 in the late phase of infant healing may be mediated by the down-regulation of the profibrotic connective tissue growth factor (CTGF), because HO-1 decreased CTGF expression at week 4 in their study (53).

Nakamura et al. (54) showed that increased induction of HO-1 exerts suppressive effects on the expression of collagen and this also counteracted changes in EMT gene changes. This depend upon a specific increase in HO-1 expression caused by cobalt protoporphyrin prevented CsA-mediated α-SMA induction, whereas genetic inhibition of HO-1 by siRNA substantially enhanced α-SMA induction compared to control cells (55). The enhanced differentiation and fibrinogenesis caused by upregulation of HO-1 expression depends on the cell specificity (56).

Taken together, the presence of Hp had no effect on the expression of HO-1 mRNA in our fibroblasts system. Our cells were not stimulated prior to infection with Hp which is consistent with the recent observation that Hp inhibits NO-stimulated HO-1 expression in gastric epithelial cells through a mechanism that requires CagA (57). Our results seem to be also consistent with the statement that Hp favors its own pathogenesis by limiting HO-1 induction (57), stimulation of fibroblast differentiation, sustaining of proliferation and matrix deposition. Another way to confirm these findings, it will be necessary to stimulate cells before subsequent infection with Hp.

We conclude, that the exposure of fibroblasts to Hp promotes their differentiation into myofibroblasts, with possible excess of matrix production in vitro. The increased expression of HSP70 mRNA suggests that induction of HSP70 confers cytoprotection against Hp infection, by inhibiting the expression of Bax. Fibroblasts seem to cooperate with epithelial component in the stomach infected with Hp, that may facilitate inflammation and peptic ulcer formation with potential risk to develop gastric cancer. However, further studies in fibroblasts affected by Hp and analysis of factors influenced by this bug at the protein level as well as the effect of incubation with various strains of Hp on the complex fibroblast-epithelial cell interaction are warranted.

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REFERENCES

9. Stein M, Bagnoli F, Halenbeck R, Rappuoli R, Fantl WJ, Covacci A. c-Src/Lyn kinases activate Helicobacter pylori...


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