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mRNA expression of EGF receptor ligands in atrophic gastritis before and after *Helicobacter pylori* eradication

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Epidermal growth factor (EGF) receptor ligands (EGFRL) including transforming growth factor alpha (TGF-α), amphiregulin, and heparin binding – EGF (HB-EGF) are involved in gastric mucosal repair in chronic gastritis. Their mRNA expression has been shown to be upregulated after *Helicobacter pylori* (H.p.)-eradication but little is known about this gene expression in atrophic gastritis. The purpose of our study was to investigate EGFRL mRNA expression in gastric mucosa of patients with atrophic gastritis before and after H.p.-eradication.

Material/Methods: Antral mucosal biopsies were obtained during endoscopy in 10 H.p. positive patients with atrophic gastritis and in 10 H.p. negative controls with intact mucosa. Total RNA of antral biopsies was extracted and RT-PCR was performed, the PCR-products being measured densitometrically. Values were compared with mRNA expressions in H.p. negative antral mucosa (n=10).

Results: Gastric biopsies revealed mRNA expression for TGF-α, amphiregulin and HB-EGF, both in H.p. positive atrophic antritis and in H.p. negative healthy mucosa. The mRNA expression of TGF-α in atrophic gastritis was significantly upregulated after H.p.-eradication, whereas that of amphiregulin did not change after this eradication. Expression of HB-EGF mRNA was higher in H.p.-infection than after H.p.-eradication or in H.p. negative healthy subjects.

Conclusions: H.p. positive atrophic gastritis is associated with differential mRNA expression of EGF receptor ligands. H.p.-eradication in this entity leads to unequal changes of these growth factor expressions compared to chronic active gastritis without atrophy.

key words: **EGF receptor • TGF-alpha • emphiregulin • HB-EGF • atrophic gastritis**

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BACKGROUND

Helicobacter pylori (H.p.) causes chronic active inflammation of the gastric mucosa in the majority of infected patients. Over long time it favors the loss of gastric glands with development of atrophic gastritis in up to 84% of patients [1]. This may be associated with the occurrence of intestinal metaplasia and increased risk for gastric cancer [2]. Various mechanisms for the pathogenesis of gastric cancer are discussed, including inflammation-related cascades of cytokines generation of reactive oxygen species (ROS), up- or downregulation of certain growth factors and their receptors, and the fundic atrophy-related impairment of acid output and reduced intraluminal acidity [3].

One of the growth factors, which are involved in epithelial cell proliferation and differentiation of the gastric mucosa, is the epidermal growth factor (EGF) [4,5], which binds to a specific cell receptor (EGF-R) [6].

Meanwhile, other EGF-homologous peptides (e.g. transforming growth factor-α – TGFα, amphiregulin and heparin-binding EGF – HB-EGF) have been detected. Some of them have been shown to be expressed in normal and cancerous gastric mucosa [7-11]. There are hints to suppose an association between EGF ligands (EGFRL) expression and H.p. infection [12]. Previously, we revealed that expressions of EGF receptor and mRNA of TGF-α, amphiregulin and HB-EGF are decreased in H.p. induced chronic active gastritis and that they are upregulated after successful eradication therapy [13].

The aim of our study was to evaluate whether similar changes of gene expressions of these growth factors are detectable in H.p. positive atrophic gastritis before and after H.p. eradication and to compare growth factor expression in atrophic gastritis with these in healthy H.p. negative intact gastric mucosa.

MATERIAL AND METHODS

Patients

10 patients with histologically proven atrophic gastritis and 10 healthy subjects with normal mucosa were endoscopically examined and biopsies were taken from the antral mucosa. H.p. status was confirmed by rapid urease test (CLO). The biopsies were immediately frozen at –80°C with liquid nitrogen until further processing for RNA extraction. In H.p. positive patients the endoscopical re-evaluation with CLO test was performed 8 weeks after successful 1-week standard anti-H.p. eradication therapy with omeprazol (20 mg/bd), clarithromycin (250 mg/bd) and amoxicillin (500 mg/td).

The study was approved by the Local Ethical Committee of Münster University and informed consent was obtained from all patients.

RNA extraction and DNA digestion

After tissue homogenisation by ultrasound (Labsonic 1510, Braun, Melsungen, Germany) total RNA was extracted with the acid guanidinium thiocyanate phenol method as described before (14). The RNA pellet was air dried and resuspended in HPLC grade water (18 mega Ohm resistance). RNA concentration was determined spectrophotometrically at 260 nm. To avoid DNA contamination RNA template was incubated with 1 U RNAase free DNAase (Promega/Serva, Heidelberg, Germany) in a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mg/ml BSA at 37°C for 15 min. Before further processing DNAase was heat inactivated at 95°C for 2 min.

Semiquantitative RT-PCR

3 µg RNA was subjected to RT-PCR for 1 h at 37°C using 20 U MuLV Reverse Transcriptase (Boehringer Mannheim, Germany) in a 5-fold buffer supplied with the enzyme (250 mM Tris-HCl, 200 mM KCl, 30 mM $MgCl_2$, 50 mM dithioerythriol, pH 8.3). The final concentration of desoxy-nucleotide triphosphates (dNTP-Mix, Promega/Serva) was 1 mM.

20 U of ribonuclease inhibitor (RNAsin, Promega/Serva) and 100 pmol random primer (Boehringer Mannheim, Germany) were added to yield a final volume of 20 µl. All substances were pipetted on ice. The cDNA obtained by reverse transcription (RT) was amplified by PCR using the following primer pairs selected with the computer program Oligo 5.0 (NBI Inc, Annapolis, MN, U.S.A.) and commercially synthesized and purified by HPLC (Biometra, Göttingen, Germany). Annealing temperatures and length of the PCR products are shown in parenthesis:

1. TGF-α
5'-Primer: 5' CGC CCT GTT CGC TCT GGG TAT
3'-Primer: 5' AGG AGG TCC GCA TGC TCA CAG
(55°C, 264 bp)

2. Amphiregulin
5'-Primer: 5' AGT CAG AGT TGA ACA GGT AGT TAA G
3'-Primer: 5' ACT GTA ATA ACA GCA ACA GCT GTG A
(53°C, 360 bp)

3. HB-EGF
5'-Primer: 5'AAA AGA AAG AAG AAA GGC AA
3'-Primer: 5'CTC CTA TGG TAC CTA AAC AT
(55°C, 290 bp)

Additionally as internal standard for RNA quality and quantity we used the pyruvate dehydrogenase sequence (PDH), an unregulated housekeeping gene [15]. Like most of the primers for EGFRL primers for PDH were placed on two different exons. The sequences of the PDH primers were:

5'-Primer: 5'GGT ATG GAT GAG GAC CTG GA
3'-Primer: 5'CTT CCA CAG CCC TCG ACT AA
(62°C, 105 bp)

These primers yielded a DNA signal at approximately 240 bp thereby conveniently allowing to monitor DNA contamination. The DNAase treatment effectively eliminated amplification of DNA, which might interfere with the quantitation of mRNA. PCR was performed in a thermocycler (Gene Ataq Controller, Pharmacia, Uppsala, Sweden). For all EGFRL optimal PCR conditions such as annealing temperatures as well as primer – and – $MgCl_2$-concentrations were established in pilot experiments and followed the procedures as described [13,16]. The denaturation step was performed at 95°C for 45 sec, annealing time was 1 min and primer extension was carried out at 72°C for 1 min. For amplification of the desired cDNA we used 2.5 U of Taq DNA polymerase (Gibco BRL, Gaithersburg, MD, U.S.A.) in 10 µl of 10-fold buffer supplied with the enzyme (20 mM Tris-HCl, 50 mM KCl, pH 8.4) to which 2 µl of 10 mM dNTP-Mix (Promega/Serva), $MgCl_2$ to a final concentration of 2 mM and 30 pmol of primers were added to yield a final volume of 100 µl.

To compare RNA expression of gastric biopsies before and after eradication therapy we performed a modified semiquantitative PCR analysis [17] in which for all primer pairs, including the housekeeping gene, the amount of PCR products was determined in 3-cycle intervals starting from cycle 24. PCR products were subjected to agarose gel electrophoresis with 1.5% agarose in Tris-acetate-EDTA-buffer (Merck, Darmstadt, Germany) as described [18]. Gels were stained with ethidium bromide (0.5 µg/ml, Boehringer, Mannheim, Germany), visualized on a UV-screen, photographed on Polaroid and photographs were saved on disk. Band intensities were measured by soft laser scanning densitometry and analysed with the computer program Image Quant Version 4.2 (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Optical densities (ODI) were determined in arbitrary units.

For each RNA sample and primer pair we obtained PCR kinetics by correlating ODI's with the cycle number. This enabled us to select one cycle for comparison within the exponential phase of the curves. For each RNA sample quantitative PCR was also performed with the PDH primer pair. ODI of specific primer pairs were quantitated relative to PDH kinetics leading to ODI-ratios, which allowed us to compare different RNA samples. This technique has been extensively evaluated and was found to have an interassay variation between 17 to 34% for the different primer pairs [16].

Identity of amplification products was verified by restriction analysis. TGF-α and HB-EGF were incubated with 3U BsuR I (MBI Fermentas, St. Leon-Rot, Germany) in a buffer containing 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl, pH 8.5 while amphiregulin was incubated with 3U Alu I (MBI Fermentas) in a buffer containing 33 mM Tris-acetate, 10 mM Mg-acetate and 66 mM K-acetate, pH 7.9 for 30 min at 37°C. Restriction fragments were separated by agarose gel electrophoresis as described above [16].

Table 1. Occurrence of mRNA expression of EGFRL in normal and atrophic gastritis

	Normal gastric mucosa(n=10)	Atrophic gastritis (n=10)
TGF-α	10	9
HB-EGF	9	10
Amphiregulin	6	4

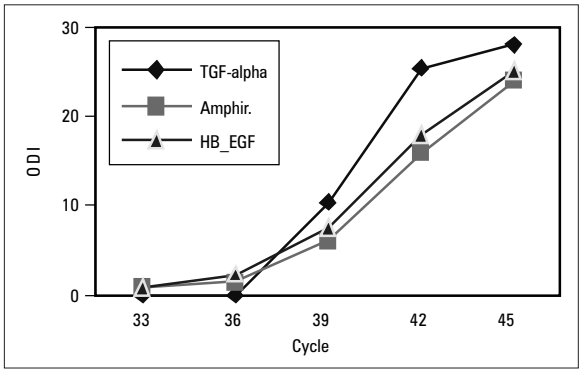

Figure 1. The mRNA expression of TGF-α, amphiregulin and HB-EGF in the antral mucosa of a patient with H.p.-positive atrophic gastritis. Data were correlated to the cycle number. Cycle 39 within the exponential phase of the curve was selected for comparison of the mRNA levels.

Statistical analysis

Data were statistically analysed with the paired Student t-test. Differences with $P<0.05$ were considered as significantly different.

RESULTS

The mRNA expression of TGF-α, amphiregulin and HB-EGF was measured in optical density units and was correlated with the cycle number (Fig. 1). Cycle 39 was chosen for mRNA determination of EGFRL and cycle 33 for determination of PDH. Optical density units were divided to obtain ODI ratios.

TGF-α and HB-EGF mRNA were found to be regularly expressed in samples of normal gastric mucosa (100 and 90%, respectively) and atrophic gastric mucosa (90 and 100%, respectively), whereas the amphiregulin expression was only detected in 60% of normal gastric mucosa and 40% of atrophic gastritis mucosa (Tab. 1).

To reveal expression of the EGFRL in a semiquantitative manner, the appearance of the amplified DNA signals was assessed relative to that of the housekeeping gene, PDH. Under our PCR conditions the amplification efficiency of the primer pairs for EGFRL was similar to that of PDH.

TGF-α mRNA expression

TGF-α mRNA expression was reduced in H.p.-infected atrophic gastritis (ODI ratio 0.12±0.06) but revealed

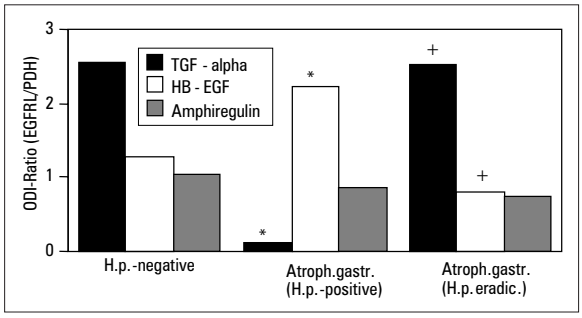

Figure 2. Levels of EGFRL mRNA expressions in normal, H.p.-infected and H.p.-eradicated atrophic gastric mucosa (ODI ratios: EGFRL/PDH). Asterisk indicates significant change as compared to values recorded in H.p. negative intact mucosa. Cross indicates significant change as compared to H.p. infected atrophic gastritis.

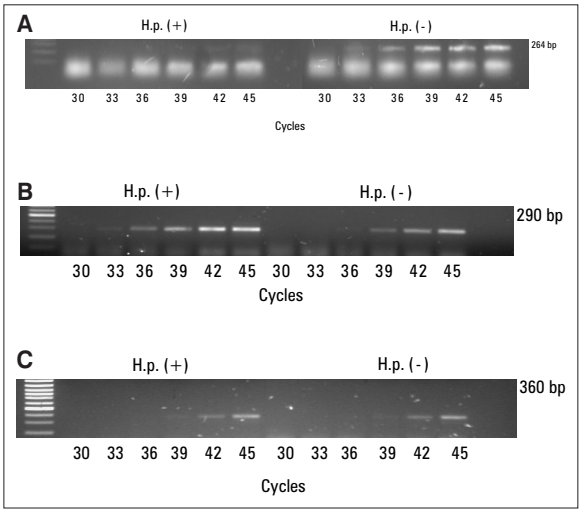

Figure 3 a-c. EGFRL-RT-PCR products of two different RNA samples of the same patient. Each RNA sample is represented on the agarose gel by 6 lanes containing PCR products from cycle numbers 30, 33, 36, 39, 42 and 45. Lanes 1-6 show the band intensities of TGF-α, HB-EGF and amphiregulin in the antral mucosa before, lanes 7-12 after eradication therapy.

significantly higher expression levels after H.p.-eradication (ODI-ratio 2.52±4.7, $p<0.01$). The latter reached approximately the mRNA expression observed in normal gastric mucosa (Fig. 2, 3a).

HB-EGF mRNA expression

The mRNA expression of HB-EGF was significantly higher in H.p.-positive atrophic gastritis (ODI ratio 2.22±1.85, $p<0.03$, Fig. 2, 3b), when compared to H.p.-negative normal mucosa (1.29±0.96). Following the H.p. eradication the mRNA of HB-EGF tended to increase but this increase did not reach statistical significance.

Amphiregulin mRNA expression

The mRNA expression of amphiregulin was similar in normal gastric mucosa (ODI ratio 1.03±1.04), H.p.-positive (ODI-ratio 0.87±1.13) and H.p.-negative (ODI-ratio 0.74±1.20) atrophic gastritis and revealed no significant differences between these three types of mucosa (Fig. 2).

Discussion

EGF and its homologous EGFRL bind to the EGF receptor, stimulate autophosphorylation by the intrinsic tyrosine activity of the receptor and induce intracellular signal transduction [19,20]. Previous immunhistochemical studies detected local expression of these growth factors in the human gastric mucosa [13,21,22]. As they are known to mediate angiogenesis [23] and stimulation of fibroblasts and smooth muscle cells [24,25], it has been suggested that they play an important role in regenerative processes of the gastrointestinal tract.

Previously, we described that mRNA expression levels of TGF-α, HB-EGF and amphiregulin are markedly decreased in the H.p.-infected stomach and increased significantly after successful H.p.-eradication to levels of mRNA expression in normal gastric mucosa [13]. In this connection antral mucosa revealed a more severe injury by H.p. infection than the oxyntic mucosa. This remains in agreement with recent study showing that H.p. eradication in duodenal ulcer patients results in normalization of EGF and EGF receptor expression in gastric mucosa after six weeks [26] or two years following eradication [27].

The aim of the study was to examine the mRNA expression of these growth factors in H.p. infected atrophic gastric mucosa before and after eradication therapy and to compare the data with the expression levels in normal gastric mucosa. The question is of special interest since reduction or reversibility of atrophic gastritis was described after eradication of H.p. [28] and since expression of growth factors such as TGF-α, and hepatocyte growth factor (HGF) may contribute to gastric cancerogenesis [29] following atrophic gastritis and gastric dysplasia.

TGF-α expression pattern in our patients with atrophic gastritis was similar to that in H.p.-induced chronic active gastritis without gastric atrophy [13]. It was markedly reduced in H.p.-infection but increased after successful eradication therapy. This finding again confirms the reports of other authors who also revealed a marked reduction in mucosal cytokine levels signifying enhanced mucosal inflammation, and increased EGF levels after H.p. eradication [26,27,30].

The mRNA expression of amphiregulin was only detected in 40% of H.p. positive atrophic gastritis and did not show significant changes after H.p. eradication. The low occurrence and unchanged expression levels independent on the H.p.-status may signify only slight influence of this growth factor on the healing process of atrophic gastritis.

Interestingly, mRNA expression of HB-EGF was higher in H.p. positive atrophic gastritis compared to normal gastric mucosa and decreased after H.p. eradication. This may hint a hyperregenerative activity, especially for stimulation of fibroblasts, in acute H.p.-infection and would agree with data of increased mucosal cell proliferation in H.p.-infected gastric mucosa [31].

It is still a matter of debate if H.p.-eradication reverses atrophy in chronic gastritis. Some authors revealed improvement of all gastritis scores including atrophy and lymphoid follicle score [32], whereas others cast doubt on the reversibility of gastric atrophy [33].

Our data emphasize that local expression of regenerative growth factors in the H.p.-positive atrophic gastritis are partially changed due to successful eradication therapy [34]. This may be associated with a possible reversibility of gastric atrophy and should be further examined in long-term follow-up studies.

CONCLUSIONS

This study shows that H.p. positive gastritis is associated with a marked upregulation TGF-α, whereas expression of mRNA for amphiregulin did not change and expression of HB-EGF was reduced after H.p. eradication as compared to that before this therapy or that in H.p. negative healthy subjects. We conclude that eradication of H.p. in atrophic gastritis is associated with differential mRNA expression of various EGFRL characterized by enhanced expression of mRNA for TGF-α, no change in expression for amphiregulin and reduced expression for HB-EGF.

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