P. C. KONTUREK [a], A. HARTWICH [b], M. ZUCHOWICZ [b], H. LABZA [b],
P. PIERZCHALSKI [c], E. KARCZEWSKA [c] W. BIELANSKI [c] , E. G. HAHN [a]
S. J. KONTUREK [c]

# HELICOBACTER PYLORI, GASTRIN AND CYCLOOXYGENASES IN GASTRIC CANCER

[a] Department of Medicine, Erlangen-Nuremberg University, Erlangen, Germany
[b] Department of Surgery, District Hospital, Cracow, Poland
[c] Department of Physiology, Jagiellonian University School of Medicine, Cracow, Poland

**Background:** Tumors arising in the stomach have worldwide distribution and the infection with *Helicobacter pylori* (*HP*) has been implicated in causation of this disease. The *HP* discovery, which is considered as the greatest advance of gastroenterology at the dawn of $3^{rd}$ millennium, is accompanied by hypergastrinemia, which seems to play a key role in gastric cancerogenesis but no study was undertaken to assess the relationship between the *HP* infection and coexpression of gastrin and cyclooxygenases (COX), the rate limiting enzymes in the eicosanoids production. **Aims:** Since gastrin is recognized as a effective gastric mitogen, it could be capable to induce COX-2, a potent tumor growth promoting and angiogenic factor, we decided 1) to compare the seroprevalence of *HP* and its cytotoxic protein, CagA, in gastric cancer patients with those in age- and gender-matched controls; 2) to determine the gene expression of gastrin and its receptors ($CCK_B$-R) in gastric cancer, 3) to assess the plasma levels, gastric lumen and tumor tissue contents of gastrin and 4) to examine the mRNA and enzyme protein expression of COX-1 and COX-2 in cancer tissue and intact gastric mucosa before and after *HP* eradication. **Material and Methods:** The trial material included 20 patients with gastric cancers and 100 age- and gender-matched controls. Anti-HP and anti-CagA IgG seroprevalence was estimated by specific antisera using ELISA tests. Gene expressions of gastrin, $CCK_B$-R, COX-1 and COX-2 was examined using RT-PCR with GAPDH as a reference and employing Western blot for COX-2 expression, while gastrin was measured by RIA. **Results:** The seroprevalence of HP, especially that expressing CagA, was significantly higher in gastric cancers than in controls. Both gastrin and $CCK_B$-R mRNA were detected by RT-PCR in the cancer tissue and similarly COX-2 mRNA and protein were found in most of cancers and in the *HP* infected antral mucosa but not in *HP* eradicated patients in whom only cancer tissue but not gastric mucosa expressed COX-2. The gastric cancer tissue contained 20 times more of immunoreactive gastrin than the *HP* infected antral gastric mucosa and following *HP* eradication the gastrin content in the tumor and antrum showed a marked and significant reduction. No significant change in $CCK_B$-R expression was noticed before and after *HP* eradication in the tumor and the corpus mucosa.
**Conclusions:** 1). Gastric carcinoma coexpresses gastrin, its receptors ($CCK_B$-R), and COX-2; 2) *HP* infection may contribute to gastric cancerogenesis *via* gastrin and

COX-2 that may account for the stimulation of tumor growth, angiogenesis, and reduction in apoptosis 3) *HP* positive patients developing gastric cancer should be considered for *HP* eradication to reduce the *HP* provoked hypergastrinemia and COX-2 overexpression in the tumor tissue.

## INTRODUCTION

Numerous epidemiological studies demonstrated that gastric malignant tumors still belong to the most common visceral malignancies with the highest death rates in Eastern Europe including Poland (1—3). There is an accumulating evidence linking gastric infection with *Helicobacter pylori* (HP) to the causation of various types of gastric pathologies including peptic ulcerations, gastric cancer and lymphomas (3—8) as well as of extragastric disorders (9, 10). Statistical evidence established a positive relationship between the occurrence of *HP* infection and gastric carcinoma (1—3).

Furthermore, an overexpression of cyclooxygenase-2 (COX-2), a rate limiting enzyme in eicosanoid production, was reported in gastric cancer by numerous investigators suggesting that this enzyme could be responsible (through excessive release of eicosanoids) for the cancer cell proliferation, angiogenesis and reduction in apoptosis (11—15).

HP is known to be accompanied by a markedly enhanced and prolonged release of gastrin (6,16) that has been suggested to account for the development of gastric cancer and to coexpress with COX-2 (18). Since *HP* infection was found to be accompanied by overexpression of immunoreactive gastrin that is a powerful mitogen for the mucosa of gastric corpus (17—19), we decided to assess the relationship between *HP* infection and gastrin release and COX-1 and COX-2 expression in gastric cancerogenesis. Since COX-2 derived prostaglandins (PG) have been suggested to stimulate angiogenesis and tumor growth (11—15), it was rationale to determine the expression of COX-2 in the gastric cancer tissue and the gastric mucosa before and after *HP* eradication.

Therefore, the aims of this study were following; 1) to compare the seroprevalence of *HP* and its cytotoxic protein, CagA, in gastric cancer patients with that in age- and gender-matched healthy controls; 2) to determine the gene expression of gastrin and its receptors ($CCK_B$-R) in gastric cancer; 3) to assess the gastrin content in the plasma and antral mucosa as well as in the tumor tissue and 4) to examine the coexpression of COX-1 and COX-2 in gastric cancer before and after *HP* eradication.

We found that the gastric cancer tissue contains higher amounts of gastrin than the antral mucosa and confirmed that cancer is capable of expressing large amounts of gastrin and gastrin-receptors (8) as well as COX-2 (18), suggesting that the immunoreactive gastrin could stimulate locally (by autocrine and paracrine pathways) the tumor cell proliferation directly or through the induction of COX-2 in cancer tissue.

## MATERIALS AND METHODS

The studies were carried out on 20 histologically verified patients as gastric carcinoma (17 men and 3 women) with the median age of 64 years (range 47 to 82 years) and 100 controls (90 men, 10 women) with the median age of 63 years (range from 47 to 82 years). Patients symptoms, age, gender, tumor site and its histology were recorded at the Department of General Surgery of Distric Hospital of Cracow, Poland and the laboratory tests were performed at the Department of Physiology, Jagellonian University School, Cracow, Poland and Laboratory of Molecular Medicine, Department of Medicine, University of Erlangen-Nuremberg, Erlangen, Germany.

The study project was reviewed and approved by local ethical committees of Distric Hospital on Cracow and University of Erlangen-Nuremberg, Germany and each studied subject signed informed consent. Most of laboratory tests were carried out in the Department of Physiology of College of Medicine, Cracow, Poland.

Before the surgery, the gastroscopy was performed to obtain mucosal biopsy from tumor tissue for histology and from the tumor tissue and the mucosa of antrum and corpus for RT-PCR analysis of gastrin and its receptors ($CCK_B$-R) and COX-1 and COX-2. Before the surgery, 10 patients obtained one week anti-HP triple therapy including Clarithromycin (500 mg bd), Amoxycillin (1000 mg bd) and Omerazole (20 mg bd) and then 10 days later the *HP* status was assessed by UBT and found to be negative in all treated patients. All patients were operated to remove the gastric tumor; the large biopsy samples were taken from the tumor and intact gastric mucosa at the remote site from the cancer for by routine histological examination to determine the stage and histological type of the tumor. The tumors were identified histologically as intestinal type (70%) and mixed type gastric carcinoma (30%) (20).

*Assessment of H. pylori and CagA status.*

The infection status was assessed by our modification of capsulated minidose $^{13}$C-Urea Breath Test (UBT) as described earlier (21) and/or by determination of IgG antibodies to *HP* by enzyme linked immunosorbent assay (ELISA) using commercially available kit (EIAGEN *H. pylori* IgG, Clone Systems, Italy). Titers higher than 15 AU/ml were considered positive (following the manufacturer recommendations). IgG antibodies against CagA were detected by ELISA using recombinant CagA kindly provided by Ora Vax Cambridge, USA as previously described (22).

*RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR) to detect mRNA for gastrin, gastrin receptors (CCK$_B$-R) and for COX-1 and COX-2.*

The samples of the tumor and macroscopically intact corpus and antral mucosa at the remote site from the tumor were taken by biopsy during surgery for determination of the mRNA expression for gastrin, CCK$_B$-R, COX-1 and COX-2 by reverse transcription-polymerase chain reaction (RT-PCR) as well as to assess the tissue content of gastrin using RIA. For this purpose two larger tumor (~100 mg) gastric antrum and corpus and two mucosa samples were taken and frozen immediately in the liquid nitrogen for the detection of the signals for gastrin and gastrin receptors as well as COX-1 and COX-2 using reverse transcriptase-polymerase chain reaction (RT-PCR) as described earlier (18, 23). Tumor tissue or intact mucosa samples were also homogenized in phosphate buffer at pH 7.7 and 0°C for 10 s with Ultra-Turrax T-25, Ika Labortechnik, Staufen, Germany. The homogenized samples were processed as for plasma gastrin RIA using gastrin antiserum No 4562 (kindly donated by Professor J.E. Rehfeld of Copenhagen, Danmark) in the final dilution of 1:280 000. The antibody used recognized G-17 and G-34 equally. The sensitivity of the present assay was 2.5 pmol/mL serum equivalent to human G-17 (16, 24) Blood samples were collected preoperatively from peripheral vein under basal conditions and after separation of the plasma they were stored at $-7$°C for gastrin RIA as described before.

RT-PCR and detection of amplified gene products were performed using the above mentioned large biopsy specimens obtained during surgery. For this purpose, total cellular RNA was isolated from these specimens using TRIzol reagent (Gibco BRL, UK). Briefly, tissue samples placed in to the liquid nitrogen and then transferred to TRIzol reagent and homogenized. After addition of chloroform the aqueous phase was separated by 13000 rpm spin in 4°C for 10 min. RNA was precipitated using isopropyl alcohol, washed with 75% ethanol and resuspended in DEPC treated water. 1 μg of total cellular RNA was used for the synthesis of cDNA in RT-PCR reaction (Promega Reverse Transcription System). The final concentration of components in 20 μl of total reaction volume was as follows; 5 mM MgCl$_2$, 1 × Reverse Transcription Buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100), 1 ml each dNTP 1 U/ml Recombinant RNasin Ribonuclease Inhibitor, 15 U/mg AMV Reverse Transcriptase 0.5 mg Oligo(dT)$_{15}$ Primer per microgram RNA. Samples were incubated at 42°C for 15 min and heated at 99°C for 5 min followed by a 5 min incubation at 0—4°C.

The PCR reactions were prepared in 1 × Dynazyme PCR buffer (Flowgen) with 40 mmol dNTPs (Pharmacia) and 10 mmol of upper and lower primers. The following primer pairs for PCR reaction were used: 5'-GCC CAG CCT CTC ATC ATC-3' as a sense primer 3'-GGG GAC AGG GCT GAA GTG — antisense for gastrin; 5'-TCT CGC GAG CTC TAC TTA GGG as sense primer and 3'-GAA GTT GCA CGT AGC AGC CA — antisense for CCK$_B$-R; 5'-AGC GGG AAA TCG TGC GTG as sense primer and 3'-CAT GCC AGT GAG CTT CCC GTT CA as antisense for GAPDH; 5'-GTC TTC ACC ACC ATG GAG AAG GCT as a sense primer; 5'ACGCCACCATTCTGTCTTTG-3' as an antisense primer for COX-1; and 5'-TCATTCACCAGGCAAATTGCTGGCAGGG-3' as a sense primer and 5'-ACAGTTCAGTCGAACGTTCTTTTAGTAGTAC-3' as an antisense primer for COX-2.

The thermal profile for all these reactions was the same; 95°C for 5 min before 40 cycles or 95°C for 45 sec, 60°C for 90 sec, and 72°C for 90 sec. This was followed by a single stage of 60°C for 120 sec and 72°C for 180 sec. PCR products were analyzed by agarose gel electrophoresis and abundance of cDNA in each sample was estimated by video densitometry analysis (Fotodyne, USA) using Gel-Pro Analyzer program.

*Protein extraction and Western blot analysis.*

Western blot analysis was performed as follows; Proteins were extracted using TRIzol reagent from the same biopsy samples used for RT-PCR analysis. Approximately 100 μg of cellular protein extracts were loaded into s well separated electrophorectically through a 10% or 13% SDS polyacrylamide gel and transferred onto Aequi-Blot ™ PVSF membrane (BioRad, USA) by electroblotting. Following protein transfer, the protein membrane was incubated in blocking buffer (0.2% I-Block, 137 mM NaCl, 20 mmol Tris-HCl, pH 7.4, 0.1% tween-20) for 1 h in room temperature. The membrane was then incubated with monoclonal mouse anti-β-actin antibody clone AC15 (dilution 1 : 5000; Sigma, USA) or goat polyclonal anti-COX-2 antibody (N-20 from Santa Cruz, USA, dilution 1 : 200). The incubation was followed by 3 washes in blocking solution for 10 min each. The appropriate secondary anti-goat IgG conjugated with alkaline phosphatase (1 : 3000; Tropix, USA) or anti-mouse IgG conjugated with alkaline phosphatase (1 : 5000; Tropix, USA) diluted in blocking solution were incubated with protein membrane for 30 min, followed by 4 washes 10 min each. The membrane was then washed $2 \times 2$ min in 1x assay buffer and the visualization solution (Tropix, USA), followed by the exposition to an X-ray film X-OMAT (Kodak, Wiesbaden, Germany).

*Data analysis*

The major groups were the *H. pylori*, cagA-seropositive and serum and gastric lavage gastrin concentrations in patients with gastric cancer and controls. In general, rank sum test, Spearman's rank test order correlation was used for relation between independent variables. A P value of less than 0.05 was accepted as significant.

## RESULTS

*Fig. 1* shows the *HP* seropositivity and CagA seropositivity in 20 gastric cancer patients and in 100 age- and gender-matched control subjects. The prevalence of *HP* seropositivity reached ∼90% in cancer but only 62% in controls and this difference in *HP* seropositivity between cancers and controls was statistically significant. The CagA seropositivity in gastric cancer patients was about thrice as high as in controls and this difference was also statistically significant (*Fig. 1*). The *HP* status checked in 10 cancer patients after triple therapy showed negative UBT in all treated patients but the *HP* and CagA seropositivity remained unchanged and these results have been omitted for the sake of clarity.
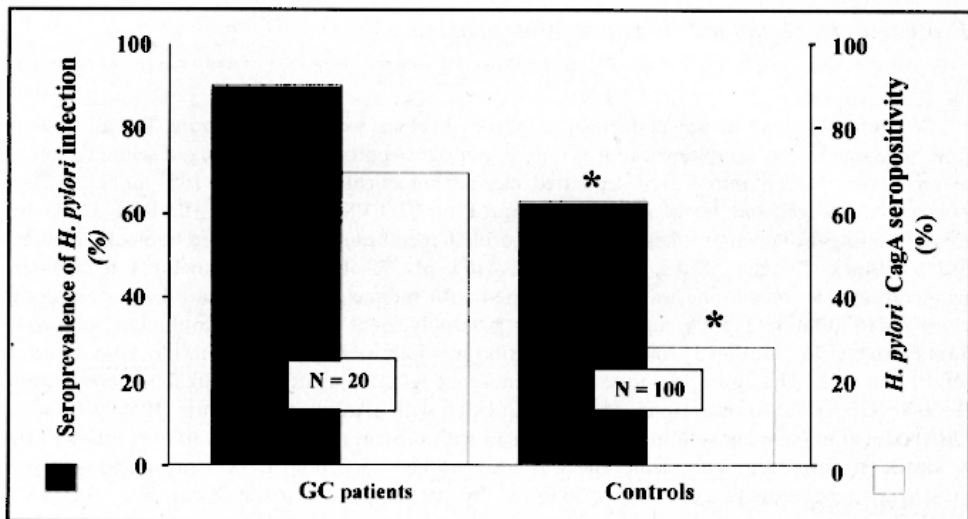
Fig. 1. H. pylori seropositivity and CagA seropositivity in 20 patients with gastric cancer and 100 controls. Mean ± SEM of 20 tests in gastric cancers and 100 controls. Asterisk indicates significant (P < 0.05) change as compared to gastric cancer patients.
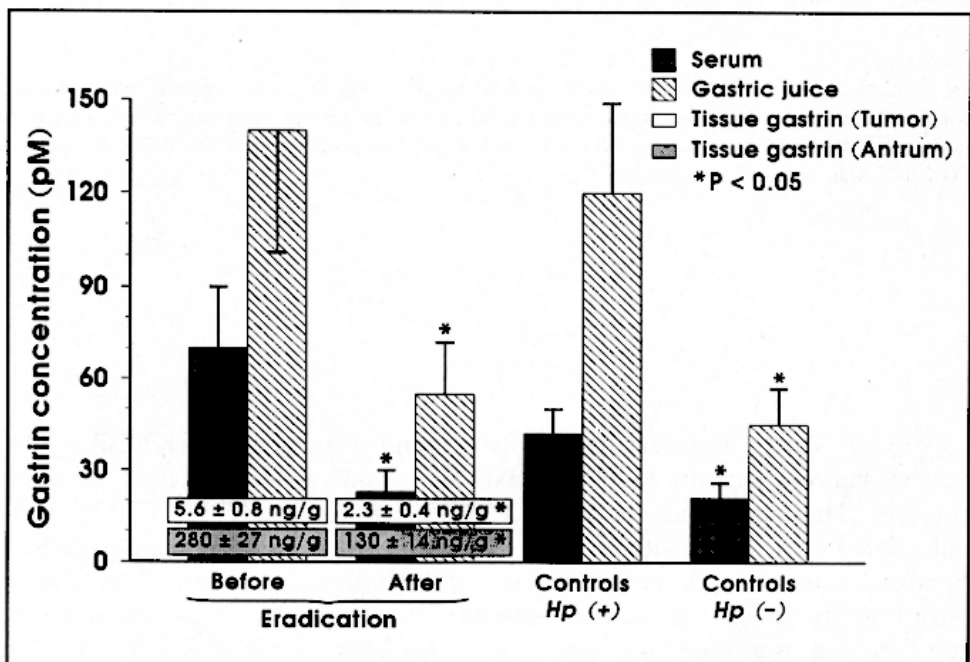


Fig. 2. Tissue (tumor and antral mucosa) and serum and gastric luminal gastrin concentrations in gastric cancer patients before and after the eradication of HP. Mean ± SEM of 20 gastrin determinations in gastric cancers and in 100 controls with or without HP infection. Asterisk indicates significant difference as compared to the values obtained in HP infected cancer patients.

Serum gastrin level in gastric cancer patients was about $71 \pm 24$ pM and this was about over twice as high as that in controls ($31 \pm 5$ pM). In 10 cancer patients, in whom serum gastrin was measured one week after triple *HP* therapy, the serum gastrin significantly decreased to about $22 \pm 3$ pM. Gastric luminal concentration, which averaged about $140 \pm 48$ pM in *HP* infected gastric cancer patients, fell after the *HP* therapy to about $55 \pm 18$ pM (*Fig. 2*). The gastrin content in gastric cancer tissue was $\sim 5.6$ ng/g of wet tissue weight and this was about 20 time more than in the *HP* infected antral mucosa and both of them significantly declined after *HP* therapy when measured at the time of gastric surgery (*Fig. 2*). This difference between the serum gastrin concentration in cancer patients and in healthy controls as well as between the serum and gastric luminal gastrin concentrations in *HP* positive and negative controls was highly statistically significant.

The tumor tissue of all cancer patients after surgery showed mRNA expression for gastrin and similar expression was detected in antral biopsy samples taken from these patients (*Fig. 3*). The mRNA expression for gastrin receptor ($CCK_B$-R) signal was observed in the tumors and in the intact gastric corpus mucosa of all tested subjects. The ratio of gastrin mRNA to GAPDH
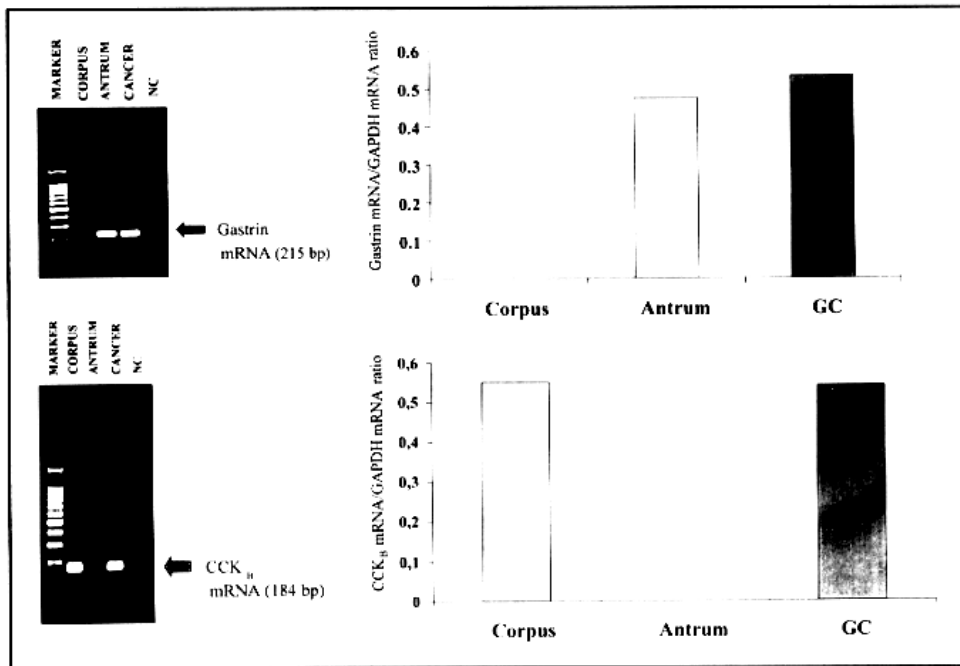


*Fig. 3.* Messenger RNA expression for gastrin (upper panel) or for its receptors ($CCK_B$-R) (lower panel) as well as the ratio of gastrin or $CCK_B$-R mRNA to GAPDH mRNA in gastric cancer (GC) tissue and *HP* infected mucosa of gastric antrum and corpus. M — marker size DNA, NC — negative control. Arrow expected PCR product (bp).

mRNA varied from case to case and it was somewhat higher, though not significantly, in the cancer tissue than in antral mucosa, while no mRNA expression of gastrin was detected in gastric corpus mucosa. The ratio of $CCK_B$-R mRNA to GAPDH mRNA was high in majority of cases in tumor tissue and reached the values similar to that in the intact corpus mucosa (*Fig. 3*). This ratio was negligible in the antrum mucosa. The mRNA expression for gastrin and $CCK_B$-R in 10 gastric tumors and gastric mucosa (antrum and corpus) before *HP* eradication were similar to those after the treatment and these results have been omitted for the sake of clarity.
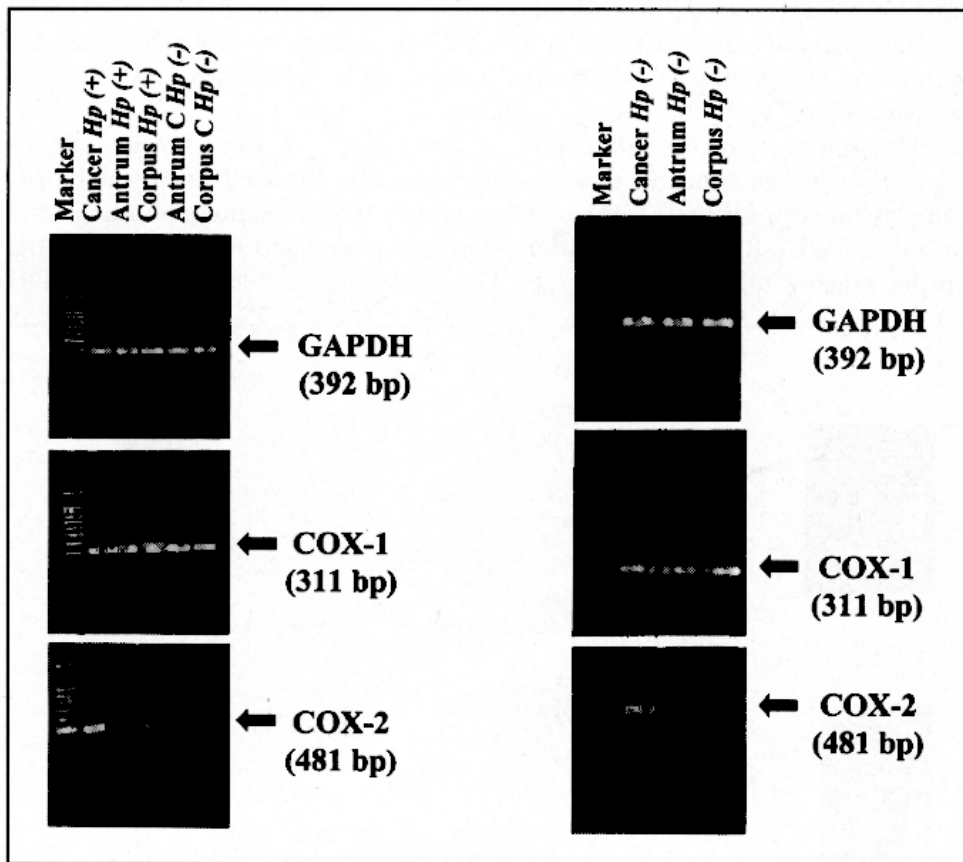


*Fig. 4*. Messenger RNA expression for COX-1 and COX-2 as well as the ratio of COX-1 or COX-2 mRNA to GAPDH mRNA in gastric cancer (GC) tissue and antrum or corpus mucosa in patients infected with *HP* or control healthy subjects without such infection. M — marker size DNA, NC — negative control. Arrow expected PCR product (bp).

*Fig. 4* shows the mRNA expression for COX-1 and COX-2 in the same cases as shown in *Fig. 3* before and after eradication of HP. The COX-1 mRNA was expressed both in the cancer tissue and in intact gastric mucosa. COX-2

mRNA expression was detected in all tissue samples of gastric cancer and to a lesser extent in the antral and corpus mucosa infected with HP. After eradication of HP, the COX-2 mRNA expression was detected only in the tumor tissue but not in antral or corpus mucosa (*Fig. 4*).

*Fig. 5* shows the protein expression for COX-2 as assessed by Western Blot in gastric cancer tissue and in antrum and corpus mucosa of patients with *HP* infection and following eradication of this infection with triple therapy. As shown, COX-2 protein was strongly expressed in the cancer tissue before and after the eradication of HP. In the gastric mucosa, the COX-2 protein was detected only in the *HP* infected patients and only in the antrum. Following *HP* eradication no COX-2 protein was expressed in the gastric mucosa.
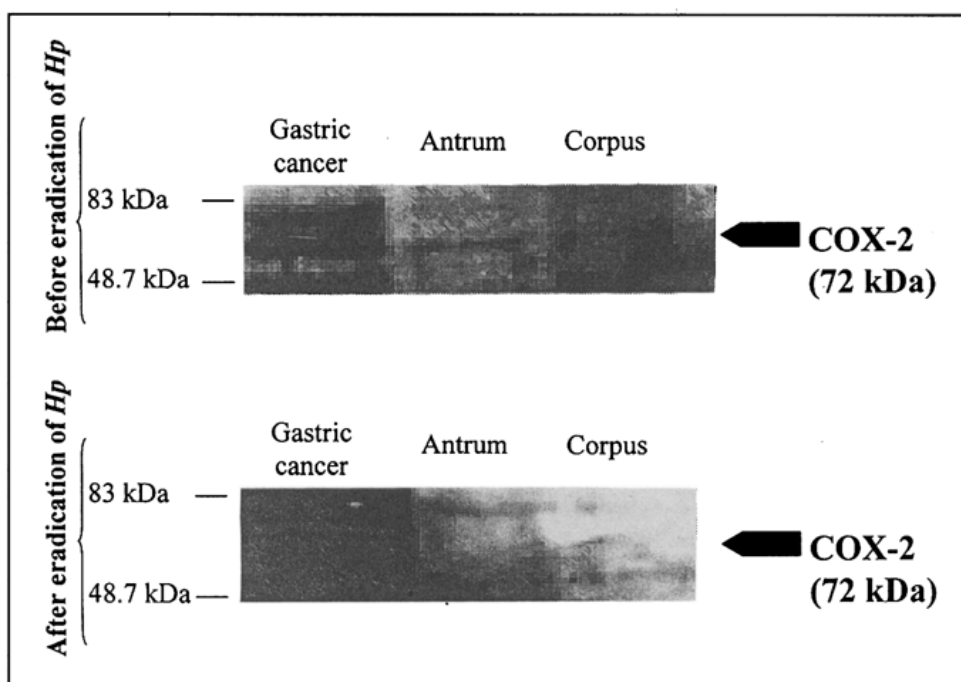


*Fig. 5.* Protein expression for COX-2 as assessed by Western blot in the gastric cancer tissue and antrum and corpus mucosa in cancer patients before and after *HP* eradication. Arrow indicates the molecular weight of the COX-2 protein.

## DISCUSSION

This study confirms previous observations that gastric neoplasma are strongly associated with *HP* and CagA seropositivity (6, 8, 25) and shows that the *HP* infection in cancer patients is accompanied by an increased mRNA

expression for gastrin and its receptors and by increased mRNA and protein expression for COX-2 in tumor tissue and in *HP* infected antral mucosa.

The reason for the higher prevalence of *HP* infection in the gastric cancer patients is not known but this infection results in a significant increase in plasma gastrin level possibly due to the impairment of local control of the G-cells by somatostatin (16) or direct stimulation of these G-cells by the histamine metabolite (Nα methyl histamine) originating from *HP* and acting from gastric lumen to stimulate the G-cells to release gastrin (26).

The question remains whether this higher *HP* infection rate plays any role in the pathogenesis of gastric cancer but the fact that it is accompanied by an increased plasma level and gastric luminal concentration of gastrin reported before (4, 6, 7, 8) and observed also in this study, suggests that this hormone could contribute to the gastric cancerogenesis by inducing higher mucosal cell proliferation, leading to atrophy and induction of COX-2 as it happens in other cancers (28, 29). Previous studies showed that gastric carcinoma exhibits increased local gastrin gene expression (8) but the gastrin plasma levels were either not measured or shown to be increased (4, 6, 8) and related to the reduction in gastric acid secretion and the removal of usual acid-dependent feedback control of the G-cells (16), suggesting that hypergastrinemia is associated with an increased risk of gastric carcinoma.

The main finding of the present study is the observation that the gastric cancer tissue expresses mRNA for gastrin and $CCK_B$-R and contains several fold higher amounts of immunoreactive gastrin than the antral mucosa. This suggests that the gastrin present in gastric cancer tissue could contribute to the increased serum levels and gastric luminal contents of this hormone supporting the major contribution of gastrin-producing cancer cells to the elevated serum and gastric luminal concentrations of hormone in gastric cancer patients. Our finding that gastric cancers exhibit higher expression and content of gastrin and its receptors reminds similar upregulation of gastrin biosynthesis described previously by our group in gastric cancers (16). As gastrin is known to be the most powerful mucosal growth promoting factor in gastrointestinal tract (18, 19), it is tempting to assume that this hormone could play a key role in the initiation and the progression of cancer in the stomach. The implication of gastrin in cancerogenesis seems to extend beyond the stomach and concerns also colorectal area and the pancreas, which originate embryologically from the same endoderm tube. This is supported by recent reports that gastrin peptides and their receptors are invariably coexpressed in the adenocarcinoma of the colon (28) and the pancreas (29). The latter study suggests that gastrin produced by pancreatic cancer cells may be an autocrine factor in carcinoma arising also from the pancreatic acinar cells. This is of particular interest because it shows that in addition to amidated gastrin, glycine-extended gastrins

as well as N-terminal progastrins were detected in the pancreatic cancer and all appear to exhibit the growth promoting effects as amidated gastrin itself.

Epidemiological studies indicate that the use of aspirin and other NSAID, whose major target is cyclooxygenase, a rate limiting enzyme converting arachidonic acid to prostanoids, decreases the incidence and mortality from colorectal cancers (30—33). COX-2 has been found to occur frequently in the colorectal cancer where it is held responsible for the production of extensive amounts of prostaglandins, tumor cell proliferation, angiogenesis, reduction in apoptosis and enhanced tumor invasiveness. Assuming that *HP* and gastrin are initiators for gastric (and colorectal or pancreatic) carcinogenesis, the excessive production of prostanoids might be implicated in promotion of this process and could be a target for the chemotherapy using specific COX-2 inhibitors (13—15, 34). Our results confirm the gene expression of both COX-1 and COX-2 in the gastric cancer and thus, supports the notion that, indeed, COX-2- and COX-2-derived prostanoids play a role in the development of gastric cancer and should be a target of treatment using specific COX-2 inhibitors. As the elimination of the initiator of cancerogenesis such as increased gastrin production may not be feasible, the inhibition of the promotion of this process, particularly of angiogenesis, tumor growth and reduction in apoptosis, could be attained by application novel anti-inflammatory drugs. Based on our data that the *HP* infection is a common infection in gastric cancer patients and leads to hypergastrinemia and overexpression of COX-2, both factors probably responsible for the promotion of gastric cancer, it seems advisable to eradicate the bacteria to prevent or reverse the gastric cancer. As shown, for the first time, in this report, the *HP* infection has a potent influence on COX-2 expression in cancer tissue and in the gastric mucosa both at the mRNA and protein levels. Eradication of *HP* results in the decrease in expression of COX-2 in cancer tissue and in the disappearance of COX-2 expression from the gastric mucosa. It may be assumed, therefore, that eradication of the *HP* should be considered as the first step after diagnosis of gastric cancer for two reasons; 1. to reduce the excessive production of gastrin and 2) to attenuate the overexpression of COX-2 to inhibit the tumor cell growth, angiogenesis and to reverse the falling apoptosis.

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Author's address: Prof. Dr S. J. Konturek, Department of Physiology, University School of Medicine, ul. Grzgorzecka 16, Cracow, Poland.