Protease-activated receptor-2 mediates expression of cyclooxygenase-2 and integrin in *Helicobacter pylori* -infected gastric epithelial cells

Ji Hye Seo

Department of Medical Science

The Graduate School, Yonsei University

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Directed by Professor Kyung Hwan Kim

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Ji Hye Seo

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This certifies that the doctoral dissertation of Ji Hye Seo is approved

Thesis Supervisor : Kyung Hwan Kim

Hyeyoung Kim : Thesis Committee Member

Min Goo Lee : Thesis Committee Member

Sung Hoon Noh: Thesis Committee Member

Yong Chan Lee: Thesis Committee Member

The Graduate School

Yonsei University

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제 말과 행동하나하나 너무도 예쁘게 봐주시는 시어른들, 항상 저의 건강을 챙겨주시고 힘들때 카운셀러도 해주신 우리 어머님, 생일때 편지도 써주시는 자상한 아버님, 부족한 며느리인데도 잘 봐주셔서 감사드립니다. 안부를 먼저 물어보고 절 너무 좋게 봐주는 우리 은정이 아가씨, 만날때마다 어진이랑 잘 놀아주고 가끔 치아관리까지 해주시는 어진이 고모부 성민씨 감사합니다. 항상 믿어주시고 제편이 되어주시는 엄마, 아빠. 부모님의 지원이 아니었으면 제가 이 자리에 있지 못했을거라 생각됩니다. 처음 이쪽길로 서겠다는 모티브가 되어준 동권이 오빠, 날 너무 좋아해주는 동국이 오빠, 올케언니 규현이 언니, 조카 윤지와 창덕이게도 감사의 말 전합

니다.

마지막으로 이 분을 만나지 못했다면 지금의 제가 없었을겁니다. 학위중에 만나 저를 끔찍히도 생각해주고 지금도 저만 보면 피로가 풀린다는 소중한 인연 한민씨에게 감사드립니다. 힘들때도 있었지만 행복을 더 많이 가져다 준 우리 아들 어진군에게도 고맙다고 전합니다.

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Ji Hye Seo

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ABSTRACT

Protease-activated receptor-2 mediates expression of cyclooxygenase-2 and integrin in *Helicobacter pylori* - infected gastric epithelial cells

Ji Hye Seo

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Kyung Hwan Kim)

Protease-activated receptor-2 (PAR-2), subgroup of G-protein coupled receptor family, is known to be associated with the expression of cyclooxygenase-2 (COX-2) and integrins. Infection of *Helicobacter pylori* (*H. pylori*) increased the expression of COX-2 and adhesion molecule such as integrin $\alpha_5\beta_1$ with enhanced cell adhesion in human gastric carcinoma cell line, AGS cells. The aim of this study is whether *H. pylori* infection induced the PAR-2 expression and its expression is related to COX-2 and integrins which were increased by *H. pylori* infection. After treatment of *H. pylori* to AGS cells at the ratio of 100:1, mRNA and protein expression of PAR-2 and integrins were determined by RT-PCR and immunoblotting. Involvement of integrin isoforms in cell adhesion was determined by adhesion assay. PAR-2 activation and induction of integrin $\alpha_5\beta_1$ were observed in *H. pylori*-infected AGS cells. *H. pylori* promoted cell adhesion through integrin $\alpha_5\beta_1$ - dependent manner in AGS cells. When AGS cells were treated with antisense oligodeoxynucleotide for PAR-2, *H. pylori*-induced expression of COX-2 and integrin $\alpha_5\beta_1$ were suppressed. The results suggest that the expression of COX-2 and integrin $\alpha_5\beta_1$ are mediated by

PAR-2 in *H. pylori* infected AGS cells. The *H. pylori*-stimulated cell adhesion to poly-L-lysine, fibronectin, collagen IV or laminin was effectively inhibited by the G_i protein blocker pertussis toxin, and Src kinase inhibitor herbimycin A. These results show that *H. pylori* regulates cellular adhesion by inducing PAR-2/G protein signalings.

In conclusion, *H. pylori* induces cell adhesion mediated by COX-2 and integrin $\alpha_5\beta_1$, which may be initiated by PAR-2 activation in AGS cells.

Key words: *Helicobacter pylori*, cyclooxygenase-2, integrin $\alpha_5\beta_1$, protease-activated receptor-2

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I. INTRODUCTION

Gastric cancer is the second largest cause of cancer-related death, responsible for upto 10% of all deaths from cancer worldwide[1]. It is now assumed that, in genetically predisposed individuals, microenvironment factors, such as dietary carcinogens and *Helicobacter pylori(H. pylori)*-related infection, produce genotypic and phenotypic changes that ultimately progress to malignant transformation[1]. Colonization of *H. pylori* leads to cell hyperproliferation within the infected mucosa and has been associated with chronic gastritis, peptic ulceration and gastric cancer[2-4]. Although pathogenic mechanisms involved in *H. pylori*-induced inflammation are not completely clarified, there is accumulating evidence that activated neutrophils play an important role in the pathogenesis of *H. pylori*-induced gastric diseases[5,6]. Several studies have demonstrated that *H. pylori* stimulated gastric hyperproliferation[7,8], an essential step in the preliminary stage for the development of gastric carcinoma. *H. pylori* induces expression of cyclooxygenase-2[9], c-fos and c-jun[10] in gastric epithelial cells.

Protease-activated receptor(PAR)s are G protein-coupled receptors that are activated by the cleavage of their N-terminal domains by proteases[11,12]. The family of PARs currently include four members: PAR-1, PAR-2, PAR-3, and PAR-4. The coagulant protease thrombin is the physiologocal activator of

PAR-1, PAR-3, and PAR-4. PAR-2 is activated by multiple trypsin-like serine proteases including trypsin, tryptase, and coagulation proteases upstream of thrombin, factors VIIa and Xa, but not by thrombin[13]. PAR-2 activation can induce G protein-mediated signal transduction, intracellular calcium mobilization, and the release of granulocyte macrophage-colony stimulating factor, interleukin-6 and interleukin-8, and prostaglandins[14]. Interleukin-1 or tumor necrosis factor-α does not affect PAR-1 expression but increases PAR-2 and PAR-4 mRNA levels by 5- and 4- fold, respectively, as determined by quantitative polymerase chain reaction[15]. Invasive breast and pancreatic cancer cells express high levels of PAR-2 compared with normal cells[16].

Previous reports described that the secretion of trypsinogen-1 and -2 by gastric[17] and ovarian cancer cells[18] and their expression in human cancer tissues of the ovary[19] and the lung[20], and in normal vasculalar endothelial cells. All these facts suggest the possibility that tumor-derived trypsin may be involved in the invasive growth of cancer cells. Trysin expresses in blood vessels surrounding tumors[21]. Furthermore, 22 suggested that PAR-2 could be activated by serum containing a little trypsin activity even to the level of 10 nM, in human pancreatic cancer cells in which PAR-2 was highly expressed whereas that was not activated in human pancreatic cancer cells in which PAR-2 was normally expressed. These reports suggested that highly expressed PAR-2 in gastric cells can be activated by a little trypsin in blood. Evidence has been accumulated to show that trypsin is produced excessively in many cancer cells of the digestive tract, including gastric cancer, and it is supposed to contribute to the growth and diffusion of cancer cells[23]. In line with this, overexpression of exogenous trypsinogen cDNA in human gastric cancer cells has been reported to increase their tumorigenicity in nude mice[24]. Trypsinogen secreted by tumor cells, if activated to trypsin, can stimulate the growth and adhesiveness of the trypsinogen-producing MKN-1 cells in an autocrine manner[25]. In addition, the MKN-1 transfectants overexpressing trypsinogen

showed high tumorigenicity in the abdominal cavity of nude mice compared with the parent MKN-1 cells[24].

Cyclooxygenase-2(COX-2) is responsible for tumor cell proliferation, angiogenesis, reduction in apoptosis, and tumor invasion in various tissues[26]. Our previouse study shows that NF-κB mediates COX-2 expression, which may be related to cell proliferation, in human gastric cancer cells[27]. COX-2 expression is transcriptionally regulated by NF-κB which is activated by reactive oxygen synthesis[28]. *H. pylori*-induced activation of NF-κB contributes to the expression of several genes involved in inflammation such as IL-8[9] and cell adhesion such as ICAM-1[29]. Cell adhesion - related gene expression such as intergrin α5 *H. pylori* in gastric epithelial AGS cells[30]. PAR-2 regulates cell proliferation and enhances COX-2 mRNA expression in human pancreatic cancer cells[31].

Integrins are heterodimeric transmembrane receptors consisting of one α and one β subunit. The two subunits collaborate to bind ligands, which are extracellular matrix proteins, including fibronectin, collagen, laminin, and vitronectin[32,33] or counter-receptor of the Ig subfamily. The most abundant, constitutive integrins in the epidermis are $\alpha_2\beta_1$(collagen receptor), $\alpha_3\beta_1$(predominantly a laminin 5 receptor) and $\alpha_6\beta_4$(laminin)[34]. $\alpha_5\beta_1$ (fibronectin receptor) and αvβ6(receptor for fibronectin and tenasin) are induced in culture and on wounding[34-37]. The adhesion of cancer cells to extracellular matrix proteins(ECMP) is thought to play an important role in invasion and metastasis[38]. 14α and 8β subunits have reported. Many subunits associate with only one subunits. The β_1 subfamily, which is identical to the very-late-activation antigen, includes the fibronectin receptor, laminin receptor and collagen receptor of epithelial cells, fibroblasts and lymphocytes.

This study was aimed to whether *H. pylori* infection induces the PAR-2 expression and its expression is related to COX-2 and adhesion molecules including integrin $\alpha_5\beta_1$ in gastric epithelial AGS cells.

II. MATERIALS AND METHODS

1. Reagents and Antibodies

Human plasma fibronectin, poly-L-lysine, murine sarcoma type IV collagen, murine sarcoma laminin, Bovine serum albumin(BSA), and soybean trypsin inhibitor (SBTI) from Sigma(St. Louis, MO, USA) pertussis toxin from and herbimycin from Calbiochem(San Diego, CA, USA). Trizol reagent from Molecular Research Center(Cincinnati, OH, USA) and synthetic peptides(SLIGRL) from Korea basic science institute(Seoul, Republic of Korea). Anti-human integrin α_5 monoclonal antibody, PAR-2, and COX-2 from Santa cruz(California,USA) anti-human integrin β_1 monoclonal antibody from serotec(Raleigh, NC, USA).

2. Cell line and Culture Condition

Human gastric carcinoma cell line AGS was obtained from the American Type Culture Collection(Manassas, VA, USA) and cultured at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. RPMI 1640(Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum(Gibco, NY, USA), 2mM glutamine, 100U/ml penicillin, and 100g/ml streptomycin(Sigma, St. Louis, MO, USA). The cells are seeded in 6-well cell culture plates at $5x10^5$cells per well in a volume of 2 ml and cultured to reach 80% confluency. Prior to stimulation, each well is washed twice with 2 ml of fresh cell culture medium containing no antibiotics. Bacterial cells are harvested, washed with phosphate buffered saline (PBS), and then resuspended in antibiotic-free cell culture medium. The bacterial cells are added to the cultured cells at a bacterium/cell ratio of 100:1 in a 3 ml volume.

3. Bacterial strain

H. pylori strain (HP99) is isolated from gastric antral mucosa obtained

from a Korean patient with duodenal ulcer. HP99 was previously identified as *cagA*, *vacA* positive strain[39]. HP99 is kindly provided from Dr. H. C. Jung (Seoul National University College of Medicine, Seoul, Korea). The bacteria is inoculated on chocolate agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) at 37℃ under microaerophilic conditions using an anaerobic chamber (BBL Campy Pouch® System, Becton Dickinson Microbiology Systems, Franklin Lakes, NJ, USA).

4. Reverse Transcription-PCR Ananlysis

Gene expression of integrin $\alpha_5\beta_1$ and COX-2 mRNAs is assessed using RT-PCR standardized by coamplifying the gene with the housekeeping gene β-actin, which served as an internal control because it must control for variation due to sample preparation. Total RNA is isolated from cells by guanidine thiocyanate extraction method[40]. Total RNA is reverse transcribed into cDNA using M-MLV reverse transcriptase and random hexamer primer (Promega, Madison, WI, USA), and used for PCR with integrin $\alpha_5\beta_1$, COX-2 and β-actin. Sequences of integrin α_5 primers are 5'-AGAGGCTGAGTACTCAGGACTCGTC-3' (forward promer) and 5'-TGGTGCAGTTGAGTCCCGTAA-3'(reverse primer), giving a 514 bp PCR product. For integrin β_1, forward primer was 5'-GGTGTCTGTAAGTGTACAGATCCGA-3' and reverse primer was 5'-TGACCACAGTTGTTACGGCAC-3', giving a 529 bp PCR product. For COX-2, forward primer is 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' and reverse primer is 5'-AGATCATCTCTGCCTGAGTATCTT-3', giving a296 bp PCR product. For PAR-2, forward primer is 5'-GATGGCACATCCCACGTC-3' and reverse primer is 5'-GGCATGTATGTGATAGGC-3', giving a 288 bp PCR product. For Trypsinogen-1, forward primer is 5'-TACCTTTGTGGCAGCTGCTC-3' and reverse primer is 5'-GATGTCATTGTTCAGAGTCTTC-3', giving a 304 bp

PCR product. For Trypsinogen-2, forward primer is 5'-TACCTTTGTTGCAGCTGCTG-3' and reverse primer is 5'-CACCACCCACTGTTCGCTG-3', giving a 157 bp PCR product. For β-actin, forward primer is 5'-ACCAACTGGGACGACATGGAG-3' and reverse primer is 5'- GTGAGGATCTTCATGAGGTAGTC-3', giving a 354 bp PCR product. The PCR is amplified by 27-30 repeat denauration cycles at 95℃ for 30 sec, annealing at 60℃ for 30 sec, and extension at 72℃for 30 sec. During the first cycle, the 95℃ step extend to 2 min, and on the final cycle the 72℃ step extend to 5min. PCR products are separated on 1.5% agarose gels containing 0.5 g/ml ethidium bromide and visualized by UV translumination.

5. Immunoblotting Ananlysis

AGS cells with *H. pylori* infection are homogenized in Tris-HCl (pH 7.4) buffer containing 0.5% Triton X-100 and protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, Indiana) for determination of integrin $\alpha_5\beta_1$, COX-2, and trypsin. Protein concentration of each sample is measured by the method of Bradford[41]. 100ug of cellular protein is loaded per lane, separated by 8-12% SDS-polyacrylamide gel electrophoresis under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Inc., Arlington Heights, Illinois, USA) by electroblotting. Membranes are blocked using 5% nonfat dry milk. integrin $\alpha_5\beta_1$, COX-2, and trypsin are detected by incubation of blots with corresponding monoclonal antibodies (Transduction Lab, San Diego, CA, USA and Calbiochem, San Diego, CA, USA) at a dilution of 1:1000 overnight at 4℃, followed by anti-rabbit and anti-mouse secondary antibodies and conjugated to horseradish peroxidase, and determination of enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) using exposure to BioMax MR film (Kodak, Rochester, NY, USA).

6. Preparation of Oligodeoxynucleotides (ODNs)

Single-stranded oligodeoxynucleotides(ODNs) are produced commercially (Gibco, NY, USA). ODNs are phosphorothioate-modified to reduce intracellular nuclease digestion. antisense(AS) and sense(S) ODNs targete the ATG start codon of the PAR-2 mRNA. The sequence of PAR-2 AS ODN is 5'-TCCGCATCCTCCTGGAA-3'. The sequence of PAR-2 S ODN is 5'-TTCCAGGAGGATGCGGA-3'.

7. Trypsin Assay

Cell culture supernatants (125 μl) are added to 1ml of assay mixture (0.5M HEPES at pH7.5, 2M NaCl, containing 5mM Z-arg AMC). The reaction is carried out 4℃ and read in model SPF-500^{TM} spectroflurometer using 360 nm excitation and 430 nm emission filters. A standard curve was generated with known concentrations of Suc-leu-leu-val-tyr-7-amino -4-methylcoumarin (AMC). Trypsin activity was expressed as nmol AMC.

8. Cell adhesion assay

Adhesion of AGS cells to extracellular matrix (ECM) proteins is assayed using the modified method of 42 et al. Each well of 96-well plates is incubated with 50 ul solution of poly-L-lysine (10 μg/ml), collagen IV (10 μg/ml), laminin (10 μg/ml), or fibronectin (10 μg/ml) (Sigma, St. Louis. MO, USA) overnight at room temperature. These wells were blocked with 100 ul of the serum-free RPMI 1640 containing 1% BSA at 37^{o}C for 1 hr and then washed with PBS. Cells cultured for 12 hr with or without *H. pylori* are detached by incubation with 0.25% trypsin containing 0.9 mM EDTA, washed with PBS, and suspened into the serum-free RPMI 1640 containing 0.02% BSA at the density of 1.5 x 10^5 cells/ml. The cell suspension (100 μl) was inoculated into each well of the ECM proteins-coated wells and incubated at 37^{o}C for 1 hr. Non-adherent cells were removed by gently

agitation, and adherent cells were stained with 0.5% crystal violet (water:methanol, 4:1). The adhesion is quantified by measuring the optical density at 600 nm.

III. RESULTS

1. Effects of PAR-2 activation on adhesion of AGS cells

It has been reported that trypsin activates PAR-2 efficiently, stimulating signal transduction through G-proteins[39,40]. Therefore, the activation of PARs seemed to be a possible mechanism for the stimulatory effect of trypsin on adhesion of AGS cells to various ECM proteins. First, the expression of PAR-2 mRNAs and protein were examined by reverse transcription-PCR analysis(RT-PCR) and immunoblotting in *H. pylori*-infected AGS cells (Fig.1).

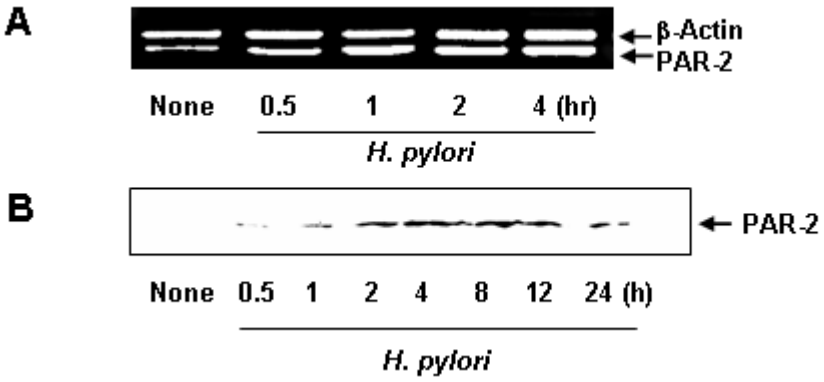


Figure 1. Expression of PAR-2 mRNA and protein in *H. pylori*-infected AGS cells. Each total RNA of AGS cells was reverse-transcribed, and transcript was amplified by PCR using primer corresponding to PAR-2 and normalized to β-actin expression(A). Whole lysates were prepared and analyzed by SDS-PAGE and immunoblotting with PAR-2 antibody(B).

2. Effect of *H. pylori* on the expression of integrin $\alpha_5\beta_1$ and COX-2 mRNA and protein in AGS cells

To confirm the expression of genes, we analyzed the levels of mRNA for these genes by coamplifying these genes with β-actin. When the time course of these genes was investigated in cells cultured with *H. pylori*, mRNA

expression of integrin $\alpha_5\beta_1$ and COX-2 was up-regulated in cells after 2 or 4 to 24 hrs of culture with *H. pylori* (Figure. 2A). When total RNAs derived from cells cultured with and without *H. pylori* were subjected to RT-PCR using primer pairs for integrin $\alpha_5\beta_1$ and COX-2 mRNA levels for these genes were increased in *H. pylori*–infected cells as compared to none-infected cells. To determine whether the levels of protein expressions for integrin $\alpha_5\beta_1$ and COX-2 genes were correlated with that of their mRNA expression in *H. pylori*-infected cells, immunoblotting studies were carried out. As shown in Figure. 2B, integrin $\alpha_5\beta_1$ and COX-2 were induced at 12 and 4 hrs respectively. These resulst suggested that *H. pylori* infection was significantly associated with the up-regulation of integrin $\alpha_5\beta_1$ and COX-2 in AGS cells.

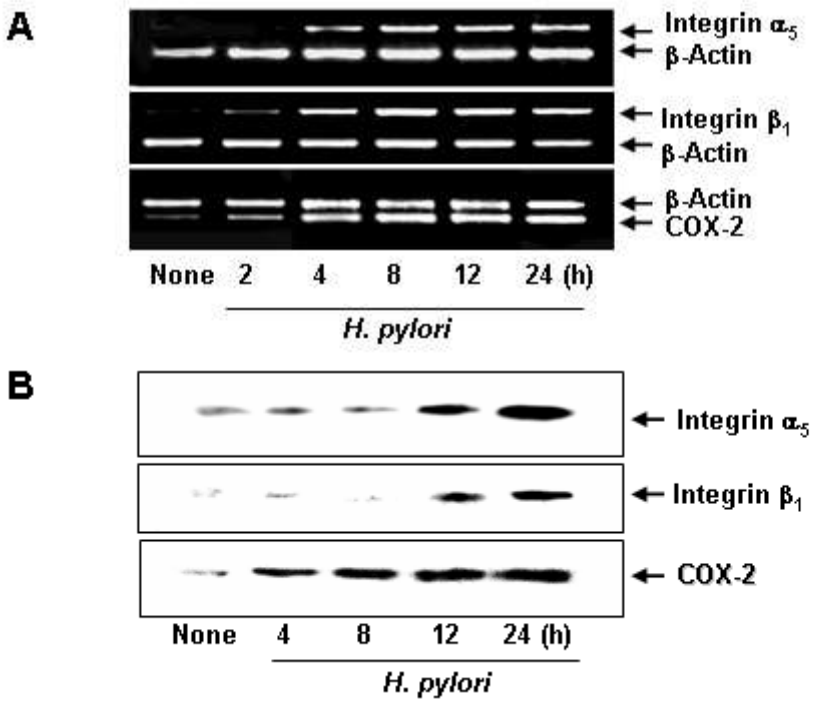


Figure 2. Time-dependent induction of integrin $\alpha_5\beta_1$ and COX-2 mRNA and protein in *H. pylori*-infected AGS cells. AGS cells were seeded in 6-well

culture plates at $5X10^5$ cells per well and cultured to reach 80% confluency. The bacterial cells were added to the cultured cells at a bacterium:cell ratio of 100:1. Total RNA was extracted, and integrin $\alpha_5\beta_1$ and COX-2 mRNA were quantified by RT-PCR and normalized to β-actin expression(A). Cells were stimulated by *H. pylori* for the indicated times. Proteins in whole cell lysates were separatedby SDS-PAGE and immunoblotting with integrin $\alpha_5\beta_1$ and COX-2 antibodies(B).

3. The role of PAR-2 in *H. pylori*-induced integrin $\alpha_5\beta_1$ and COX-2 expression

To investigate the direct effect of PAR-2 on integrin $\alpha_5\beta_1$ and COX-2, antisense oligodeoxynucleotide(AS ODN) for PAR-2 was transfected into the *H. pylori*-infected AGS cells and integrin $\alpha_5\beta_1$ and COX-2 mRNA and protein levels were determined. To address a potential role for PAR-2 in mediating *H. pylori*-induced DNA synthesis, AS ODN to PAR-2 was introduced into cells using DOTAP as a transfection agent. RT-PCR analysis demonstrated that mRNA expression of integrin $\alpha_5\beta_1$ and COX-2 was substantially reduced in the cells transfected with AS ODN for PAR-2 (Figure. 3A). Expression of PAR-2 in *H. pylori*-infected AGS cells was reduced in part following transfection of PAR-2 AS ODN as determined by immunoblotting using a PAR-2 antibody(Figure. 3B). The results clearly demonstrated that *H. pylori*-induced integrin $\alpha_5\beta_1$ and COX-2 expression is mediated by PAR-2 in AGS cells. Figure. 3C showed that *H. pylori* stimulates integrin $\alpha_5\beta_1$-mediated adhesion of AGS cells to poly-L-lysine, fibronectin, collagen type IV and laminin through PAR-2 activation. To confirm this possibility, we examined that enhancement of cell adhesion to extracelluar matrix proteins by *H. pylori* was inhibited in the cells transfected with PAR-2 AS ODN. This suggests that *H. pylori*-stimulated cell adhesion is mediated by the activation of PAR-2.

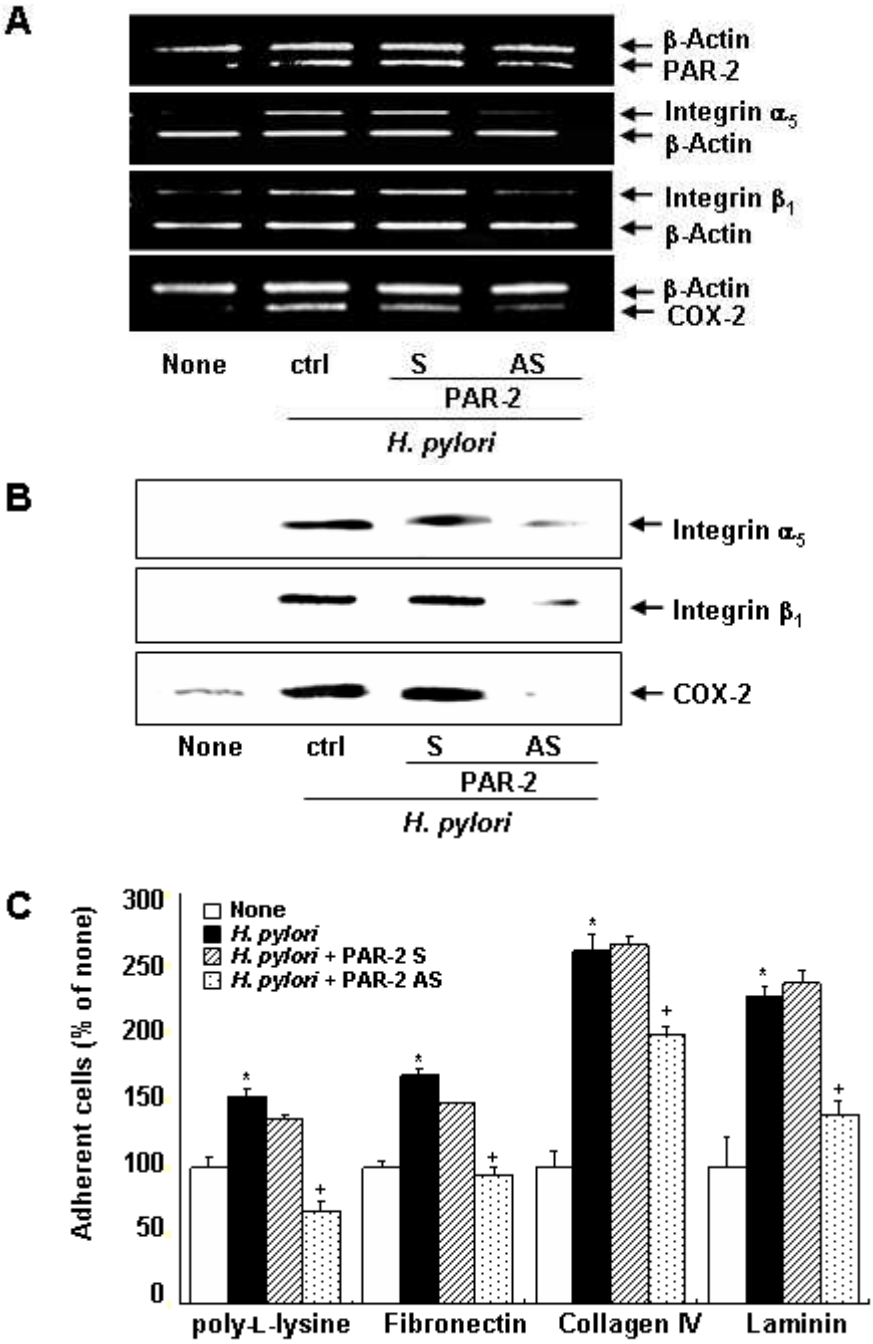


Figure 3. Integrin $\alpha_5\beta_1$ and COX-2 expression in *H. pylori*-infected AGS cells with transfection of S or AS ODN for PAR-2 and adhesion assay. AGS cells

were seeded in 6-well culture plates at $5X10^5$ cells per well transfected with S or AS ODNs for PAR-2 for 15hr. The bacterial cells were added to the cultured cells at a bacterium/cell ratio of 100:1 for 4 hr. Total RNA was extracted, and integrin $\alpha_5\beta_1$ and COX-2 mRNAs were quantified by RT-PCR and normalized to β-actin expression(A). Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotting with integrin $\alpha_5\beta_1$ and COX-2 antibodies(B). Blots are each representatives of results from three similar independent experiments. For adhesion assay of *H. pylori*-infected AGS cells(C), AGS cells were cultured for 12 hr with or without (none) *H. pylori*. Cells were suspended in serum free-RPMI 1640 containing 0.02% BSA and plated in 96 well plates precoated with the indicated ECM proteins. After 1 hr-incubation, adherent cells were determined by the absorbance of the stained cells. Hundred percent indicates the absorbance of the cells which were cultured without *H. pylori* (none). Results are expressed as means±S.E. of four separate experiments. $^{*}p<0.05$ compared to the corresponding none. $^{+}p<0.05$ compared to the corresponding *H. pylori*.

4. *H. pylori*-induced trypsin acting via PAR-2 increases COX-2, and integrin $\alpha_5\beta_1$ production

To examine the effect of PAR-2 activator, trypsin, AGS cells with or without *H. pylori* were measured trypsin concentration. As shown in Figure. 6, trypsin activity peaked at a concentration of 23 nM in *H. pylori*-infected AGS cells regardless of medium 10% FBS. *H. pylori* increased COX-2 and integrin $\alpha_5\beta_1$ mRNA levels and protein expression(Figure. 2). Trypsin action is mediated by its specific receptor PAR-2, because the overexpressed PAR-2 gene - transfected AGS cells also increased COX-2, integrin $\alpha_5\beta_1$ mRNA (Figure. 4A) and protein (Figure. 4B) expression. To demonstrate integrin $\alpha_5\beta_1$-mediated

adhesion of AGS cells to poly-L-lysine, fibronectin, collagen type IV and laminin through PAR-2 activation(Figure. 4C), it is examined that overexpressed PAR-2 gene - transfected AGS cells increased cell adhesion to several extracellular matrix proteins in cells cultured without *H. pylori*

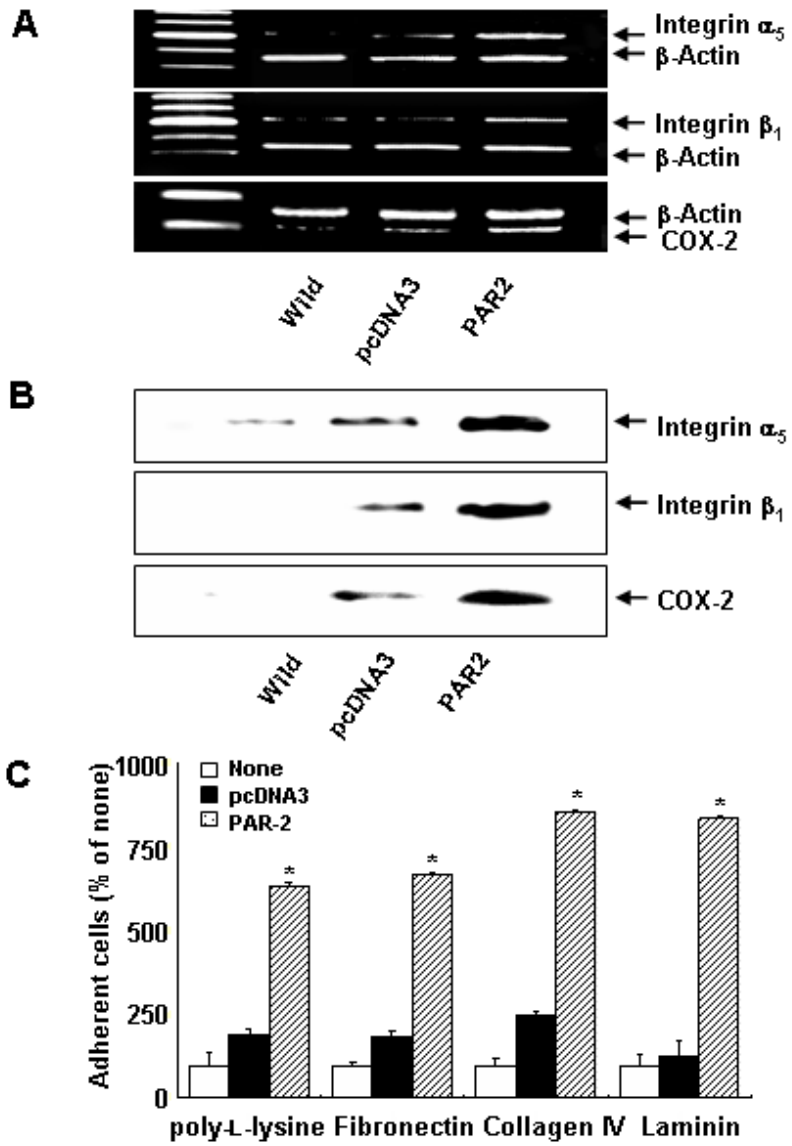


Figure 4. Integrin $\alpha_5\beta_1$ and COX-2 expression and adhesion assay in AGS cells transfected with PAR-2 overexpressed gene. AGS cells were seeded in 6-well culture plates at $5X10^5$ cells per well transfected with PAR-2 overexpressed gene for 15 hr. Total RNA was extracted, and integrin $\alpha_5\beta_1$ and COX-2 mRNAs were quantified by RT-PCR and normalized to β-actin expression(A). Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotting with integrin $\alpha_5\beta_1$ and COX-2 antibodies(B). Blots are each representatives of results from three similar independent experiments. For adhesion assay of AGS cells(C), AGS cells were cultured for 12 hr without *H. pylori*(none). Cells were suspended in serum free-RPMI 1640 containing 0.02% BSA and plated in 96 well plates precoated with the indicated ECM proteins. After 1 hr-incubation, adherent cells were determined by the absorbance of the stained cells. Hundred percent indicates the absorbance of the cells which were cultured without *H. pylori* (none). Results are expressed as means±S.E. of four separate experiments. $^*p<0.05$ compared to the corresponding none.

5. Expression of trypsinogen mRNA and trypsin protein

Expression of trypsinogen-1 and trypsinogen-2 mRNA in *H. pylori*-infectedAGS cells was determinedby RT-PCR analysis. By laser scanning of ethidium bromide-stained gel, trypsinogen- 1 and trypsinogen- 2 cDNA product of 304bp and 157bp were highly and consistently detected in *H. pylori*-infected AGS cells(Figure. 5A). *H. pylori* increased trypsin protein expression which is measured by immnoblotting(Figure. 5B).

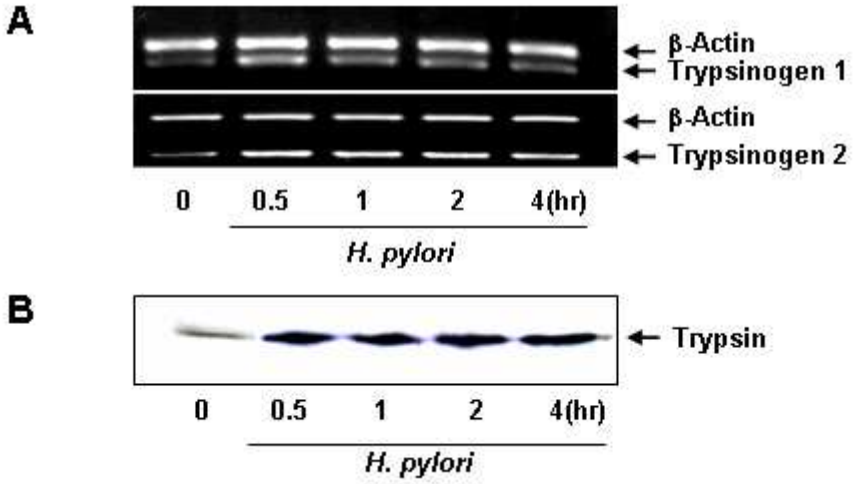
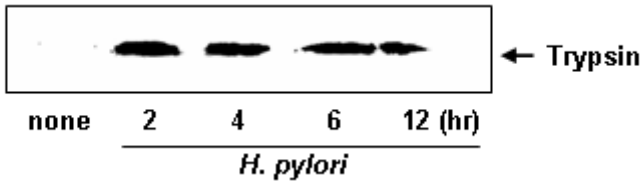


Figure 5. ***H. pylori*-induced trypsinogen and trypsin expression.** Total RNA from AGS cells was reverse-transcribed and amplified using specific primers for cationic trypsinogen (trypsinogen-1) and anionic trypsinogen (trypsinogen-2)(A). PCR products were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide. β-actin gene expression was used to confirm that equal amounts of RNA were used in each RT-PCR reaction. Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotting with anti-trypsin antibody(B). Time dependent enzyme production by *H. pylori* as analysed by immunoblotting with antibody against human trypsin.

6. Trypsin secretion and activity

To study the role of trypsin in the cell adhesion, trypsin secretion was isolated from the *H. pylori*-cultured medium. Time course of enzyme production by *H. pylori* as analysed by immunoblotting with antibody against human trypsin. The mediums were collected after removal of serum(Figure. 6A). Trypsin concentration is measured in *H. pylori*-cultured medium and none (Figure. 6B).

A



B

	Trypsin in medium(nM)
Serum free medium:	
Medium alone	3.0 ± 0.6
AGS cells in medium	5.0 ± 0.6
AGS cells with *H. pylori* in medium	23.4 ± 0.8
Serum containing medium:	
Medium alone	5.1 ± 0.6
AGS cells in medium	5.6 ± 0.7
AGS cells with *H. pylori* in medium	23.3 ± 2.1

Figure 6. *H. pylori*-induced trypsin secretion. Time dependent enzyme production by *H. pylori* was analysed by immunoblotting with antibody against human trypsin(A). The mediums were collected after removal of serum. Trypsin concentration in *H. pylori* - cultured medium are stated(B).

7. Identification of trypsin released on *H. pylori* infection

Because the trypsin-like activity in the *H. pylori*-infected AGS cells activated PAR-2, the next step was to identify the activating trypsin. To show that the trypsin activity from the *H. pylori*, the samples were treated with SBTI for 1 hr before treatment with sample loading buffer and SDS/PAGE. *H. pylori*-induced integrin $\alpha_5\beta_1$ and COX-2 mRNA expression were inhibited by SBTI treatment, compared to controls(Figure. 7A). Immunoblotting analysis using integrin $\alpha_5\beta_1$ and COX-2 confirmed that trypsin has a important role for

H. pylori-induced integrin $\alpha_5\beta_1$ and COX-2 protein expression(Figure. 7B). To demonstrate integrin $\alpha_5\beta_1$-mediated adhesion of AGS cells to poly-L-lysine, fibronectin, collagen type IV and laminin through PAR-2 activation via trypsin, It is examined that *H. pylori*-infected AGS cells with SBTI reduced cell adhesion to several extracellular matrix proteins (Figure. 7C).

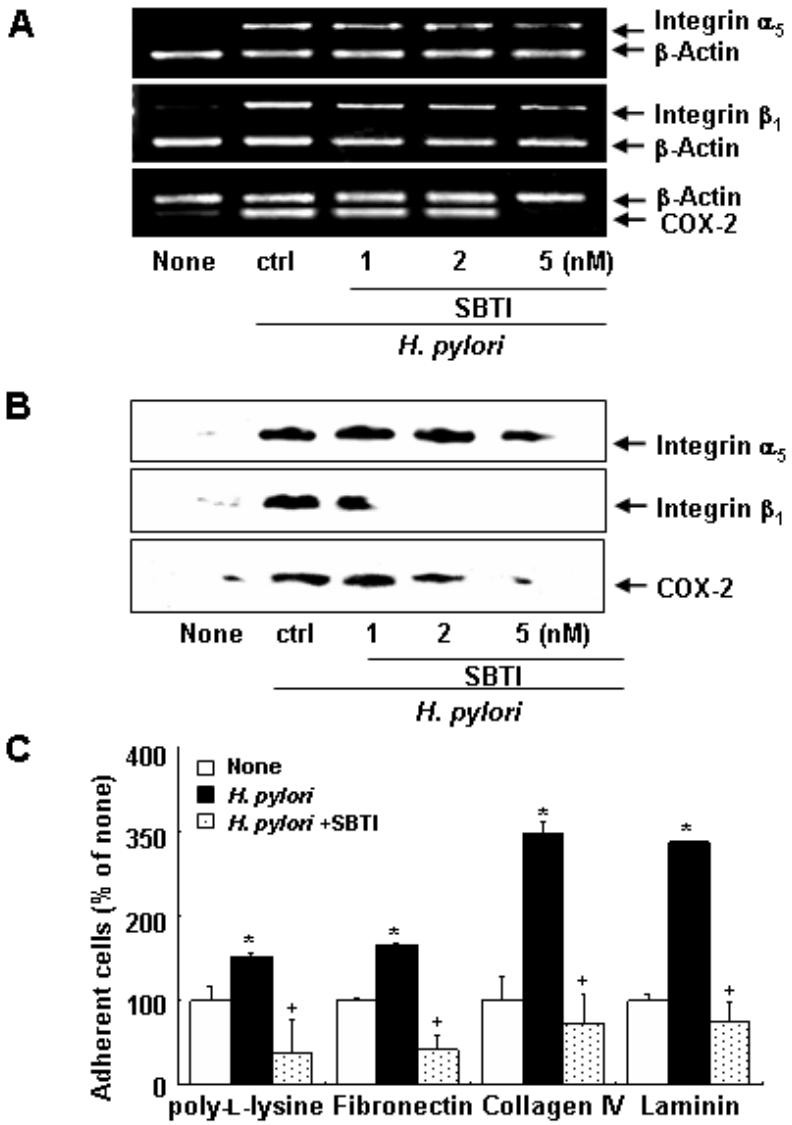


Figure 7. Soybean trypsin inhibitor(SBTI) attenuates *H. pylori*-induced integrin $\alpha_5\beta_1$ and COX-2 expression. Figure A and B, quiescent AGS cells in 6-well culture plates were preexposed (1hr) to the indicated concentrations of soybean trypsin inhibitor(SBTI), then exposed to *H. pylori* for 4 or 12 hr, repectively. Integrin $\alpha_5\beta_1$ and COX-2 mRNA expression were quantified by RT-PCR and normalized to β-actin expression(A). Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotting with integrin $\alpha_5\beta_1$ and COX-2 antibodies(B). Blots are each representatives of results from three similar independent experiments. For adhesion assay (C), cells were suspended in serum free-RPMI 1640 containing 0.02% BSA and plated in 96 well plates precoated with the indicated extracellular matrix proteins. After 1 hr-incubation, adherent cells were determined by the absorbance of the stained cells. Hundred percent indicates the absorbance of the cells which were cultured without *H. pylori* (none). Results are expressed as means±S.E. of four separate experiments. $^{*}p<0.05$ compared to the corresponding none. $^{+}p<0.05$ compared to the corresponding *H. pylori*.

8. Effect of *H. pylori* on cell adhesion to extracellular matrix proteins

To determine whether increased expression of integrin $\alpha_5\beta_1$ by *H. pylori* infection was involved in the enhancement of cell adhesion to extracelluar matrix proteins, cell adhesion to several extracellular matrix proteins in cells cultured with and without *H. pylori* is examined(Figure. 3C). When cells cultured with and without *H. pylori* were suspended and then incubated for 1 hr on wells coated with poly-L-lysine, fibronectin, collagen type IV and laminin, *H. pylori* infection significantly increased cell adhesion to poly-L-lysine and fibronectin, collagen type IV and laminin. To evaluate the contribution of integrin $\alpha_5\beta_1$ to the adhesion of cells to poly-L-lysine, fibronectin, collagen

type IV and laminin, inhibitory experiments were performed using monoclonal antibody against intergrin $\alpha_5\beta_1$. In the presence of anti-integrin α_5 and anti-integrin β_1 antibody, cell adhesion to poly-L-lysine and fibronectin were decreased in cells cultured with and without *H. pylori* (Figure. 8A,B,C,D). These results indicate that enhancement expression of integrin $\alpha_5\beta_1$ by *H. pylori* infection play an essential role in the increased cell adhesion in *H. pylori*-infected cells. Each value represents the means±S.E. (*bar*) for triplicate assays. $^{*}p<0.05$ compared to the corresponding none. $^{+}p<0.05$ compared to the corresponding *H. pylori*.

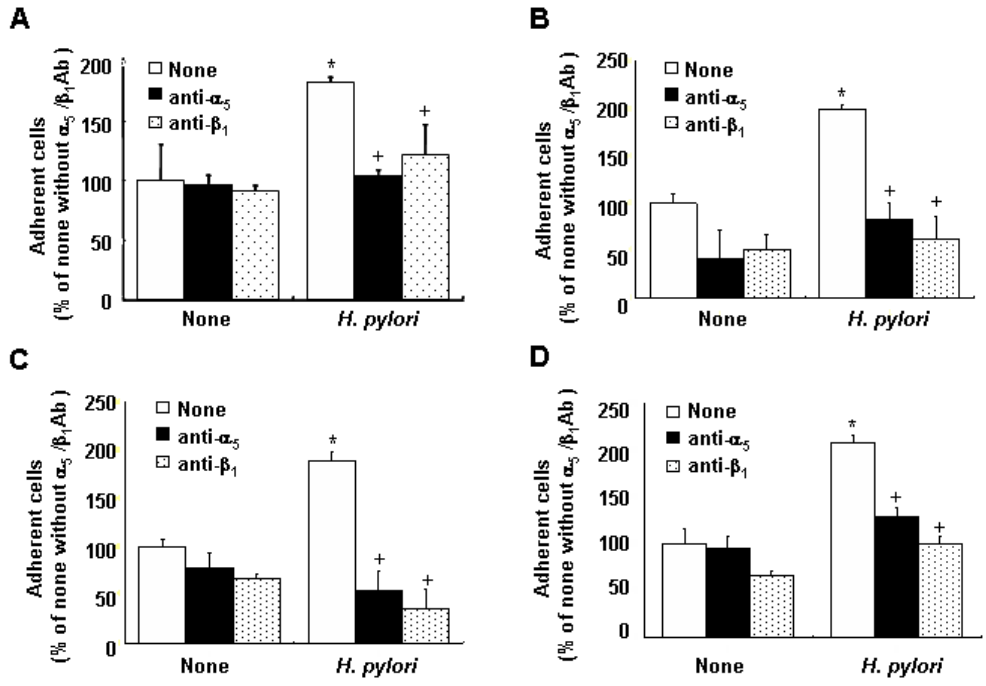


Figure 8. Effects of anti-integrin α_5, anti-integrin β_1, monoclonal antibodies on adhesion of AGScells treated with *H. pylori* to poly-L-lysine(A), fibronectin(B), collagen IV(C) and laminin(D). Confluent AGS cells were incubated without or with *H. pylori* for 15hr. AGS cells were suspended in RPMI 1640 containing 0.02% BSA and incubated without or with anti-integrin α_5 monoclonal antibody

and anti-integrin β_1 monoclonal antibodies at room temperature for 1 hr. Adhesion of these AGS cells to plastic plates precoated with 10 μg/ml poly-L-lysine (A) or 10 μg/ml fibronectin (B) 10 μg/ml collagen IV (C) 10 μg/ml laminin (D) were determined. Each value represents the means±S.E. (*bar*) for triplicate assays. $^{*}p<0.05$ compared to the corresponding none. $^{+}p<0.05$ compared to the corresponding *H. pylori*.

9. PAR-2-dependent signaling pathway in *H. pylori*-infected AGS cells

G proteins are heterotrimers consisting of α, β and γ subunits, and the subunit is grouped into four classes on the basis of sequence homology: G_s, G_i, G_q, and G_{12}[41]. PAR-1-dependent or other GPCR signalings are often inhibited by pertussis toxin, which inactivates by ADP-ribosylation the subunits of the G_i class such as three forms of α_i (α_{i1}, α_{i2}, and α_{i3}) and two forms of α_0 (α_{01} and α_{02})[42,43]. To examine the involvement of G_i proteins in the *H. pylori*-stimulated cell adhesion, effect of pertussis toxin on the stimulation of the AGS cell adhesion by *H. pylori* was investigated in this study. The stimulatory effects of *H. pylori* on adhesion of AGS cells to poly-L-lysine (Figure. 9A), fibronectin(Figure. 9B), collagen IV(Figure. 9C) and laminin(Figure. 9D) were completely blocked by pertussis toxin. It is well known that the Src family nonreceptor tyrosine kinases play important roles in PAR-1-dependent and other GPCR signalings[44,45]. Using the Src kinase inhibitor herbimycin A, It is examined that the contribution of Src kinase in the stimulatory effects of *H. pylori* on adhesion of AGS cells. Herbimycin A completely inhibited the stimulatory effects of *H. pylori* on both cell adhesions to poly-L-lysine, fibronectin, collagen IV and laminin (Figure. 9A,B,C,D). These results showed that GPCR signaling and Src kinase are involved in the stimulation of both integrin $\alpha_5\beta_1$-dependent cell adhesions by PAR-2.

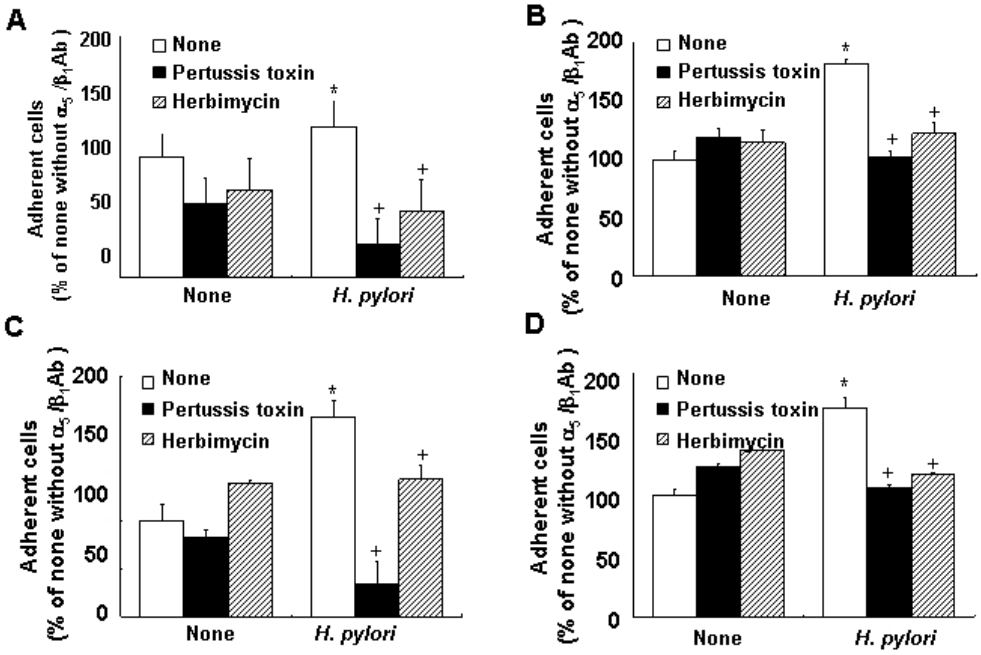


Figure 9. Effect of pertussis toxin and herbimycin A on adhesion of AGScells stimulated with *H. pylori* to poly-L-lysine(A), fibronectin(B), collagen IV(C) and laminin(D). AGS cells were incubated at room temperature without or with 400pertussis toxin for 1or 10μM herbimycin A for 2 hr. These AGS cells were incubated without (None) or with *H. pylori* for 15 hr. Adhesion of these AGS cells to plastic plates precoated with 10 μg/ml poly-L-lysine (A) or 10 μg/ml fibronectin (B) 10 μg/ml collagen IV (C) 10 μg/ml laminin (D) were determined. Each value represents the means±S.E. (*bar*) for triplicate assays. $^{*}p<0.05$ compared to the corresponding none. $^{+}p<0.05$ compared to the corresponding *H. pylori*.

IV. DISCUSSION

The main finding of this study is the discovery of a PAR-2 that displays activation in response to *H. pylori. H. pylori* stimulated the cyclooxygenase-2(COX-2) and integrin $\alpha_5\beta_1$- dependent adhesion of human gastric cancer cells to poly-L-lysine, fibronectin, collagen IV and laminin which require the activation of PAR-2. This is the first report to demonstrate a functional link between *H. pylori* and COX-2, integrin $\alpha_5\beta_1$- dependent cell adhesion involving PAR-2.

Many studies have demonstrated that active trypsin, produced an secreted by cancer cells, plays an essential role in facilitating invasion and metastasis by digesting components of the extracellular matrix[17,24,46]. However, few reports have focused on the contribution of PAR-2 protein in human gastrointestinal tract carcinomas, and on the effect of active trypsin on the growth of cancer themselves. Low concentrations of exogenous pancreatic trypsin stimulates the growth of the pancreatic cancer cell line AsPC-1 through PAR-2 activation in vitro[22]. Cell types that express PAR-2 in the intestinal tract include epithelial cells, sensory neurons, fibroblasts, mast cells, smooth muscle, and endothelial cells[47]. Trypsin and PAR-2 activating peptide AP2(SLIGKV) induce proliferation of PAR-2-positive human colon and gastric cancer cells[25,48]. For this study, we hypothesized that trypsin, possibly released from *H. pylori* itself, would be responsible for the activation of PAR-2, because bacteria is known to contain serine proteinases[49,50] and strains of *E. coli* secretes the serine proteinases EspP[51]. Moreover, other studies have shown that bacterial proteinases, such as porphyromonas gingivalis gingipains, can activate PAR-2[52]. 53 first analysed frozen tissues for levels of trypsin by zymography. Zymography revealed overexpression of trypsin-1 in colorectal cancer tissues. These results suggest that secreted latent trypsin (trypsinogen) is activated to active trypsin by autoactivation[54] or by endogenous activators, such as enterokinase, in colorectal

cancer tissues. The extracellular pH in cancerous tissue is known to be lower than that in normal tissue[55]. Trypsinogen-1 has been shown to be spontaneously converted to an active form under the acidic conditions that prevail in the extracellular space between cancer cells[55,56]. It has been reported that trypsinogen-1 produced by human colon and pancreatic cancer cells under acidic conditions is spontaneously activated and acquires gelatinolytic activity[54,57].

From the present results, it may be concluded that these characteristics of the trypsinogen expressing *H. pylori*-infected AGS cells depend on the PAR-2 activation by the self-produced trypsin. Many types of human cancer cell lines secrete trypsin in an active or inactive form in culture[58], and *in vivo* expression of trypsin is associated with malignant potential of tumor cells in gastric and ovarian cancers[58,59]. Taken together, tumor-derived trypsin seems to contribute to growth, invasion, and metastasis of human cancer cellsnot only by proteolysis of surrounding ECM proteins but also by activation of PAR-2. In gastric cancer cells, it was recently showed that trypsinogen secreted by tumor cells, when activated to trypsin, can stimulate the growth and adhesiveness of the producer cells in autocrine manner[25]. Furthermore, recent studies have shown that trypsin is a ubiquitous enzyme. It is expressed in various epithelial tissues including the gastrointestinal tract, kidney, liver, airway and skin, endothelial cells, leukocytes, and neuronal cells[60]. Importance of locally secreted trypsin at the vicinity of gastric tumor cells was recently emphasized. Normal epithelial cells surrounding gastric cancer cells are likely a source of active trypsin[60] and blood vessels surrounding tumors also express trypsin[21]. Some human gastric cancer cell lines have been shown to produce and secete trypsinogen[24,58].

PAR-2-mediated signaling is triggered by the activation of the receptor, a process that requires the cleavage of the amino-terminal exodomain of PAR-2, and generation of a new amino-terminal sequence that binds to the core

receptor and serves as a tethered ligand[13]. As compared with the PAR-1-dependent signaling, the PAR-2-dependent signaling has poorly been understood. PAR-2 can be activated by multiple enzymes, including trypsin and mast cell tryptase. As trypsin is produced in excess within the gastric cancer tissue and overexpression of trypsin is associated with enhanced gastric tumorigenicity in xenograft models[23,61]. Like activated PAR-1, activated PAR-2 stimulates generation of inositol triphosphate and mobilization of intracellular Ca^{2+} [62,63] activity of mitogen-activated protein kinases ERK-1/-2[64], and cell growth[65] in some cell lines. 66 Showed that PAR-2 peptide 2F-LIGRLO also strongly induced the nuclear translocation p65-NF-κB. PAR-2 expression was significantly correlated to depth of wall invasion, lymphatic invasion, venous invasion, and liver metastasis[67]. We think that PAR-2 plays also one of the important roles in the invasion of cancer cells. Of the many factors secreted by tumor cells, the one proteolytic enzyme, trypsin, has been correlated to the stage and type of carcinoma and is associated with cell invasion and extracellular matrix degradation[68,69].

Cyclooxygenase(COX) utilizes arachidonic acid as a substrate to produce a metastable intermediate product, which is then further processed to different prostanoids. Recently, it was shown that COX-2 induced synthesis and release of cytokines such as IL-1ß, tumour necrosis factor (TNF)-α and IL-8[70]. Also, IL-1ß and TNF-α induced de-novo synthesis of COX molecules[71,72]. This was paralleled by an enhancement of PGE_2 release into the conditioned media of these cultures and an increased COX-2 protein expression. *H. pylori* extract induces nuclear factor-κB, activator protein-1, and COX-2 in esophageal epithelial cells[73]. Now we have shown that, in AGS cells, *H. pylori* strongly induced COX-2 protein, which was barely detectable in non-stimulated cells. It has been speculated that SBTI would interfere at the mRNA and protein level with the induction of COX-2. *H. pylori*-induced release of COX-2 is

down-regulated by soybean trypsin inhibitor(SBTI), through an inhibition of COX-2 expression. It is likely that SBTI directly or indirectly inhibits biosynthesis of inducible COX-2. As previously reported[66] PARs are capable of stimulating of COX-2-drived PGI_2 release emphasizes the pathophysiological significance of PAR-1 and PAR-2 activation by circulating proteases and suggests that they contributes to the early inflammatory response by transiently increasing COX-2 expression and PGI_2 synthesis.

Integrin expression in cancer tissues demonstrates its possible contribution to tumor progression, invasion and metastasis[74]. Increased adhesiveness of tumor cells by *H. pylori* is thought to be important for their metastasis. It has been reported that the expression of integrin subunits such as integrin α_2, α_3, α_5 and α_6 was correlated with cell adhesion, invasion and metastasis of gastric carcinoma cells[25,75,76]. Increased adhesiveness of tumor cells is thought to be important for their metastasis. *H. pylori*-induced up-regulation in expression of integrin αM and αX in Kato 3 cells[77] and integrin α_7 in AGS cells[78] were reported. This integrin activation is supposed to depend on the conformational change of the integrin molecule induced by the interaction of its cytoplasmic domain with some GPCR signaling molecules[79]. However, little is known about PAR-2-dependent regulation of cell adhesion. In the present study, the stimulatory effects of trypsin and the PAR-2 ligand on adhesion of *H. pylori*-infected AGS cells to extracellular matrix proteins, including poly-L-lysine, fibronectin, collagen IV, laminin were almost completely blocked by the $G_i$inhibitor pertussis toxin. This implies that the activations of integrin $\alpha_5\beta_1$ by the PAR-2-stimulated signaling is mediated by different G proteins.

The important role of the Src family tyrosine kinases in GPCR signalings has been reported in many cell types[42,44]. In accordance with these studies, the present study showed that Src kinase plays an essential role in the regulation of integrin $\alpha_5\beta_1$ by the trypsin/PAR-2 signaling. Many GPCRs induce

Ras-dependent mitogenic signals, leading to the activation of the mitogen-activated protein kinase cascade[45]. Recent studies have shown that some GPCR mitogenic signalings transactivate two types of tyrosine kinases, receptor tyrosine kinases such as epidermal growth factor receptor and platelet-derived growth factor receptor, and integrin-associated FAKs such as $p125^{FAK}$ and Pyk2[44,45]. The stimulation of cell adhesion and cell growth by the trypsin/PAR-2 signaling found in this study are well consistent with the above model of GPCR signaling. A possibility is that PAR-2 may be induced by *H. pylori*, because *H. pylori*-associated gastric atropy and intestinal metaplasia are considered as precancerous lesions of the stomach[80,81].

It is first found PAR-2 expression in *H. pylori*-infected gastric epithelial AGS cells. PAR-2 is capable of stimulating COX-2 and integrin $\alpha_5\beta_1$ which emphasize the pathophysiological significance of PAR-2 activation by *H. pylori*-induced circulating trypsin. Inhibition of PAR-2 synthesis could represent a new and promising way to contain COX-2 / integrin signaling might contribute to cell adhesion, invasion and possibly cell metastasis in gastric epithelial AGS cells.

V. CONCLUSION

The present study identified a novel interaction between *H. pylori* and PAR-2, and characterized the role of PAR-2 on *H. pylori*-induced COX-2 and integrin $\alpha_5\beta_1$ expression using molecular, biochemical approaches to conclude:

1. *H. pylori* induces the expression of PAR-2, COX-2, and integrin α_5,β_1 in AGS cells.

2. Trypsin is constitutively expressed in AGS cells and the expression is increased by *H. pylori* infection.

3. *H. pylori*-induced PAR-2 activation mediates expression of COX-2 and integrin α_5,β_1, which is associated with adhesion of AGS cells to ECM proteins.

From these results, it could be concluded that *H. pylori* upregulates constitutive PAR-2 expression, and activation of PAR-2 induces COX-2 and integrin α_5,β_1-dependent adhesion of human gastric epithelial AGS cells.

REFERENCES

1. Hohenberger P, Gretschel S. Gastric cancer. Lancet 2003;362(9380):305-315.

2. Blaser MJ. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J Infect Dis 1990;161(4):626-633.

3. Negrini R, Savio A, Poiesi C, Appelmelk BJ, Buffoli F,Paterlini A, et al. Antigenic mimicry between *Helicobacter pylori* and gastric mucosa in the pathogenesis of body atrophic gastritis. Gastroenterology 1996;111(3):655-665.

4. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, et al. *Helicobacter pylori* infection and the risk of gastric carcinoma. N Engl J Med 1991;325(16):1127-1131.

5. Yoshida N, Granger DN, Evans DJ, Jr., Evans DG, Graham DY, Anderson DC, et al. Mechanisms involved in *Helicobacter pylori*-induced inflammation. Gastroenterology 1993;105(5):1431-1440.

6. Blaser MJ. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. Gastroenterology 1992;102(2):720-727.

7. Bechi P, Balzi M, Becciolini A, Maugeri A, Raggi CC, Amorosi A, et al. *Helicobacter pylori* and cell proliferation of the gastric mucosa: possible implications for gastric carcinogenesis. Am J Gastroenterol 1996;91(2):271-276.

8. Peek RM, Jr., Moss SF, Tham KT, Perez-Perez GI, Wang S, Miller GG, et al. *Helicobacter pylori cagA*+ strains and dissociation of gastric epithelial cell proliferation from apoptosis. J Natl Cancer Inst 1997;89(12):863-868.

9. Kim H, Lim JW, Kim KH. *Helicobacter pylori*-induced expression of interleukin-8 and cyclooxygenase-2 in AGS gastric epithelial cells: mediation by nuclear factor-κB. Scand J Gastroenterol 2001;36(7):706-716.

10. Meyer-ter-Vehn T, Covacci A, Kist M, Pahl HL. *Helicobacter pylori* activates mitogen-activated protein kinase cascades and induces expression of the proto-oncogenes c-fos and c-jun. J Biol Chem 2000;275(21):16064-16072.

11. Nystedt S, Emilsson K, Wahlestedt C, Sundelin J. Molecular cloning of a potential proteinase activated receptor. Proc Natl Acad Sci U S A 1994;91(20):9208-9212.

12. Xu WF, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, et al. Cloning and characterization of human protease-activated receptor 4. Proc Natl Acad Sci U S A 1998;95(12):6642-6646.

13. Hou L, Kapas S, Cruchley AT, Macey MG, Harriott P, Chinni C, et al. Immunolocalization of protease-activated receptor-2 in skin: receptor activation stimulates interleukin-8 secretion by keratinocytes in vitro. Immunology 1998;94(3):356-362.

14. Coughlin SR, Camerer E. PARticipation in inflammation. J Clin Invest 2003;111(1):25-27.

15. Hamilton JR, Frauman AG, Cocks TM. Increased expression of protease-activated receptor-2 (PAR-2) and PAR-4 in human coronary artery by inflammatory stimuli unveils endothelium-dependent relaxations to PAR2 and PAR4 agonists. Circ Res 2001;89(1):92-98.

16. Kamath L, Meydani A, Foss F, Kuliopulos A. Signaling from protease-activated receptor-1 inhibits migration and invasion of breast cancer cells. Cancer Res 2001;61(15):5933-5940.

17. Koshikawa N, Yasumitsu H, Umeda M, Miyazaki K. Multiple secretion of matrix serine proteinases by human gastric carcinoma cell lines. Cancer Res 1992;52(18):5046-5053.

18. Miyagi E, Yasumitsu H, Hirahara F, Minaguchi H, Koshikawa N, Miyazaki K, et al. Characterization of matrix-degrading proteinases and their inhibitors secreted by human gynecological carcinoma cells. Jpn J Cancer Res 1995;86(6):568-576.

19. Hirahara F, Miyagi Y, Miyagi E, Yasumitsu H, Koshikawa N, Nagashima Y, et al. Trypsinogen expression in human ovarian carcinomas. Int J Cancer 1995;63(2):176-181.

20. Kawano N, Osawa H, Ito T, Nagashima Y, Hirahara F, Inayama Y, et al. Expression of gelatinase A, tissue inhibitor of metalloproteinases-2, matrilysin, and trypsin(ogen) in lung neoplasms: an immunohistochemical study. Hum Pathol 1997;28(5):613-622.

21. Koshikawa N, Nagashima Y, Miyagi Y, Mizushima H, Yanoma S, Yasumitsu H, et al. Expression of trypsin in vascular endothelial cells. FEBS Lett 1997;409(3):442-448.

22. Ohta T, Shimizu K, Yi S, Takamura H, Amaya K, Kitagawa H, et al. Protease-activated receptor-2 expression and the role of trypsin in cell proliferation in human pancreatic cancers. Int J Oncol 2003;23(1):61-66.

23. Compton SJ, Sandhu S, Wijesuriya SJ, Hollenberg MD. Glycosylation of human proteinase-activated receptor-2 (hPAR-2): role in cell surface expression and signalling. Biochem J 2002;368(Pt 2):495-505.

24. Miyata S, Miyagi Y, Koshikawa N, Nagashima Y, Kato Y, Yasumitsu H, et al. Stimulation of cellular growth and adhesion to fibronectin and vitronectin in culture and tumorigenicity in nude mice by overexpression of trypsinogen in human gastric cancer cells. Clin Exp Metastasis 1998;16(7):613-622.

25. Miyata S, Koshikawa N, Yasumitsu H, Miyazaki K. Trypsin stimulates integrin alpha(5)beta(1)-dependent adhesion to fibronectin and proliferation of

human gastric carcinoma cells through activation of proteinase-activated receptor-2. J Biol Chem 2000;275(7):4592-4598.

26. Fu S, Ramanujam KS, Wong A, Fantry GT, Drachenberg CB, James SP, et al. Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. Gastroenterology 1999;116(6):1319-1329.

27. Lim JW, Kim H, Kim KH. Nuclear factor-κB regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells. Lab Invest 2001;81(3):349-360.

28. Kim H, Seo JY, Roh KH, Lim JW, Kim KH. Suppression of NF-κB activation and cytokine production by N-acetylcysteine in pancreatic acinar cells. Free Radic Biol Med 2000;29(7):674-683.

29. Mori N, Wada A, Hirayama T, Parks TP, Stratowa C, Yamamoto N. Activation of intercellular adhesion molecule 1 expression by *Helicobacter pylori* is regulated by NF-kappaB in gastric epithelial cancer cells. Infect Immun 2000;68(4):1806-1814.

30. Lim JW, Kim H, Kim KH. Cell adhesion-related gene expression by *Helicobacter pylori* in gastric epithelial AGS cells. Int J Biochem Cell Biol 2003;35(8):1284-1296.

31. Yada K, Shibata K, Matsumoto T, Ohta M, Yokoyama S, Kitano S. Protease-activated receptor-2 regulates cell proliferation and enhances cyclooxygenase-2 mRNA expression in human pancreatic cancer cells. J Surg Oncol 2005;89(2):79-85.

32. Hynes RO. Integrins: a family of cell surface receptors. Cell 1987;48(4):549-554.

33. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992;69(1):11-25.

34. Watt FM. Studies with cultured human epidermal keratinocytes: potential relevance to corneal wound healing. Eye 1994;8 (Pt 2):161-162.

35. Breuss JM, Gallo J, DeLisser HM, Klimanskaya IV, Folkesson HG, Pittet JF, et al. Expression of the beta 6 integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. J Cell Sci 1995;108 (Pt 6):2241-2251.

36. Zambruno G, Marchisio PC, Marconi A, Vaschieri C, Melchiori A, Giannetti A, et al. Transforming growth factor-beta 1 modulates beta 1 and beta 5 integrin receptors and induces the de novo expression of the alpha v beta 6 heterodimer in normal human keratinocytes: implications for wound healing. J Cell Biol 1995;129(3):853-865.

37. Hakkinen L, Hildebrand HC, Berndt A, Kosmehl H, Larjava H. Immunolocalization of tenascin-C, alpha9 integrin subunit, and alphavbeta6 integrin during wound healing in human oral mucosa. J Histochem Cytochem 2000;48(7):985-998.

38. Liotta LA, Rao CN, Wewer UM. Biochemical interactions of tumor cells with the basement membrane. Annu Rev Biochem 1986;55:1037-1057.

39. Seo JH, Lim JW, Kim H, Kim KH. *Helicobacter pylori* in a Korean isolate activates mitogen-activated protein kinases, AP-1, and NF-kappaB and induces chemokine expression in gastric epithelial AGS cells. Lab Invest 2004;84(1):49-62.

40. Chomczynski P, Mackey K. Substitution of chloroform by bromo-chloropropane in the single-step method of RNA isolation. Anal Biochem 1995;225(1):163-164.

41. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.

42. Murakami KI, Tsuboi Y, Bessho K, Yokoe Y, Nishida M, Iizuka T. Outcome of arthroscopic surgery to the temporomandibular joint correlates with stage of internal derangement: five-year follow-up study. Br J Oral Maxillofac Surg 1998;36(1):30-34.

43. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 1991;64(6):1057-1068.

44. Santulli RJ, Derian CK, Darrow AL, Tomko KA, Eckardt AJ, Seiberg M, et al. Evidence for the presence of a protease-activated receptor distinct from the thrombin receptor in human keratinocytes. Proc Natl Acad Sci U S A 1995;92(20):9151-9155.

45. Simon MI, Strathmann MP, Gautam N. Diversity of G proteins in signal transduction. Science 1991;252(5007):802-808.

46. Chung J, Gao AG, Frazier WA. Thrombspondin acts via integrin-associated protein to activate the platelet integrin alphaIIbbeta3. J Biol Chem 1997;272(23):14740-14746.

47. LaMorte VJ, Harootunian AT, Spiegel AM, Tsien RY, Feramisco JR. Mediation of growth factor induced DNA synthesis and calcium mobilization by Gq and Gi2. J Cell Biol 1993;121(1):91-99.

48. Della Rocca GJ, Maudsley S, Daaka Y, Lefkowitz RJ, Luttrell LM. Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. J Biol Chem 1999;274(20):13978-13984.

49. Luttrell LM, Daaka Y, Lefkowitz RJ. Regulation of tyrosine kinase cascades by G-protein-coupled receptors. Curr Opin Cell Biol 1999;11(2):177-183.

50. Koivunen E, Ristimaki A, Itkonen O, Osman S, Vuento M, Stenman UH. Tumor-associated trypsin participates in cancer cell-mediated degradation of extracellular matrix. Cancer Res 1991;51(8):2107-2112.

51. Vergnolle N. Modulation of visceral pain and inflammation by protease-activated receptors. Br J Pharmacol 2004;141(8):1264-1274.

52. Darmoul D, Marie JC, Devaud H, Gratio V, Laburthe M. Initiation of human colon cancer cell proliferation by trypsin acting at protease-activated receptor-2. Br J Cancer 2001;85(5):772-779.

53. Paetzel M, Dalbey RE, Strynadka NC. The structure and mechanism of bacterial type I signal peptidases. A novel antibiotic target. Pharmacol Ther 2000;87(1):27-49.

54. Rawlings ND, Barrett AJ. Families of serine peptidases. Methods Enzymol 1994;244:19-61.

55. Brunder W, Schmidt H, Frosch M, Karch H. The large plasmids of Shiga-toxin-producing Escherichia coli (STEC) are highly variable genetic elements. Microbiology 1999;145 (Pt 5):1005-1014.

56. Lourbakos A, Potempa J, Travis J, D'Andrea MR, Andrade-Gordon P, Santulli R, et al. Arginine-specific protease from Porphyromonas gingivalis activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. Infect Immun 2001;69(8):5121-5130.

57. Yamamoto H, Iku S, Adachi Y, Imsumran A, Taniguchi H, Nosho K, et al. Association of trypsin expression with tumour progression and matrilysin expression in human colorectal cancer. J Pathol 2003;199(2):176-184.

58. Ohta T, Tajima H, Fushida S, Kitagawa H, Kayahara M, Nagakawa T, et al. Cationic trypsinogen produced by human pancreatic ductal cancer has the characteristics of spontaneous activation and gelatinolytic activity in the presence of proton. Int J Mol Med 1998;1(4):689-692.

59. Stubbs M, McSheehy PM, Griffiths JR, Bashford CL. Causes and consequences of tumour acidity and implications for treatment. Mol Med Today 2000;6(1):15-19.

60. Tajima H, Ohta T, Elnemr A, Yasui T, Kitagawa H, Fushida S, et al. Enhanced invasiveness of pancreatic adenocarcinoma cells stably transfected with cationic trypsinogen cDNA. Int J Cancer 2001;94(5):699-704.

61. Oyama K, Ohta T, Nishimura GI, Elnemr A, Yasui T, Fujimura T, et al. Trypsinogen expression in colorectal cancers. Int J Mol Med 2000;6(5):543-548.

62. Miyata S, Koshikawa N, Higashi S, Miyagi Y, Nagashima Y, YanomaS, et al. Expression of trypsin in human cancer cell lines and cancer tissues and its tight binding to soluble form of Alzheimer amyloid precursor protein in culture. J Biochem (Tokyo) 1999;125(6):1067-1076.

63. Kato Y, Nagashima Y, Koshikawa N, Miyagi Y,Yasumitsu H, Miyazaki K. Production of trypsins by human gastric cancer cells correlates with their malignant phenotype. Eur J Cancer 1998;34(7):1117-1123.

64. Koshikawa N, Hasegawa S, Nagashima Y, Mitsuhashi K, Tsubota Y, Miyata S, et al. Expression of trypsin by epithelial cells of various tissues, leukocytes, and neurons in human and mouse. Am J Pathol 1998;153(3):937-944.

65. Naef M, Yokoyama M, Friess H, Buchler MW, Korc M. Co-expression of heparin-binding EGF-like growth factor and related peptides in human gastric carcinoma. Int J Cancer 1996;66(3):315-321.

66. Vergnolle N. Proteinase-activated receptor-2-activating peptides induce leukocyte rolling, adhesion, and extravasation in vivo. J Immunol 1999;163(9):5064-5069.

67. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1-2):55-63.

68. Belham CM, Tate RJ, Scott PH, Pemberton AD, Miller HR, Wadsworth RM, et al. Trypsin stimulates proteinase-activated receptor-2-dependent and -independent activation of mitogen-activated protein kinases. Biochem J 1996;320 (Pt 3):939-946.

69. Mirza H, Yatsula V, Bahou WF. The proteinase activated receptor-2 (PAR-2) mediates mitogenic responses in human vascular endothelial cells. J Clin Invest 1996;97(7):1705-1714.

70. Syeda F, Grosjean J, Houliston RA, Keogh RJ, Carter TD, Paleolog E, et al. Cyclooxygenase-2 induction and prostacyclin release by protease-activated receptors in endothelial cells require cooperation between mitogen-activated protein kinase and NF-kappaB pathways. J Biol Chem 2006;281(17):11792-11804.

71. Fujimoto D, Hirono Y, Goi T, Katayama K, Hirose K, Yamaguchi A. Expression of protease activated receptor-2 (PAR-2) in gastric cancer. J Surg Oncol 2006;93(2):139-144.

72. Koivunen E, Saksela O, Itkonen O, Osman S, Huhtala ML, Stenman UH. Human colon carcinoma, fibrosarcoma and leukemia cell lines produce tumor-associated trypsinogen. Int J Cancer 1991;47(4):592-596.

73. Walz DA, Fenton JW. The role of thrombin in tumor cell metastasis. Invasion Metastasis 1994;14(1-6):303-308.

74. Kobayashi H, Shinohara H, Gotoh J, Fujie M, Fujishiro S, Terao T. Anti-metastatic therapy by urinary trypsin inhibitor in combination with an anti-cancer agent.Br J Cancer 1995;72(5):1131-1137.

75. Casey ML, Cox SM, Beutler B, Milewich L, MacDonald PC. Cachectin/tumor necrosis factor-alpha formation in human decidua. Potential role of cytokines in infection-induced preterm labor. J Clin Invest 1989;83(2):430-436.

76. Spaziani EP, Lantz ME, Benoit RR, O'Brien WF. The induction of cyclooxygenase-2 (COX-2) in intact human amnion tissue by interleukin-4. Prostaglandins 1996;51(3):215-223.

77. Abdel-Latif MM, Windle H, Terres A, Eidhin DN, Kelleher D, Reynolds JV. *Helicobacter pylori* extract induces nuclear factor-kappa B, activator protein-1, and cyclooxygenase-2 in esophageal epithelial cells. J Gastrointest Surg 2006;10(4):551-562.

78. Giancotti FG, Mainiero F. Integrin-mediated adhesion and signaling in tumorigenesis. Biochim Biophys Acta 1994;1198(1):47-64.

79. Koike N, Todoroki T, Komano H, Shimokama T, Ban S, Ohno T, et al. Invasive potentials of gastric carcinoma cell lines: role of alpha 2 and alpha 6 integrins in invasion. J Cancer Res Clin Oncol 1997;123(6):310-316.

80. Ura H, Denno R, Hirata K, Yamaguchi K, Yasoshima T. Separate functions of alpha2beta1 and alpha3beta1 integrins in the metastatic process of human gastric carcinoma. Surg Today 1998;28(10):1001-1006.

81. Cox JM, Clayton CL, Tomita T, Wallace DM, Robinson PA, Crabtree JE. cDNA array analysis of *cag* pathogenicity island-associated *Helicobacter pylori* epithelial cell response genes. Infect Immun 2001;69(11):6970-6980.

82. Sepulveda AR, Tao H, Carloni E, Sepulveda J, Graham DY, Peterson LE. Screening of gene expression profiles in gastric epithelial cells induced by *Helicobacter pylori* using microarray analysis. Aliment Pharmacol Ther 2002;16 Suppl 2:145-157.

83. Brass LF, Manning DR, Cichowski K, Abrams CS. Signaling through G proteins in platelets: to the integrins and beyond. Thromb Haemost 1997;78(1):581-589.

84. Konturek PC, Bielanski W, Konturek SJ, Hahn EG. *Helicobacter pylori* associated gastric pathology. J Physiol Pharmacol 1999;50(5):695-710.

85. Kato M, Asaka M, Shimizu Y, Nobuta A, Takeda H, Sugiyama T. Relationship between *Helicobacter pylori* infection and the prevalence, site and histological type of gastric cancer. Aliment Pharmacol Ther 2004;20 Suppl 1:85-89.

ABSTRACT (IN KOREAN)

*H. pylori*에 감염된 위 상피세포에서 cyclooxygenase-2와 integrin 발현에 protease-activated receptor-2의 역할

지도교수 김경환

연세대학교 대학원 의과학과

서지혜

Protease-activated receptor-2(PAR-2)는 G단백 결합 수용체 중 하나이며 COX-2 나 인티그린 발현에 깊은 관련이 있다. *H. pylori* (*H. pylori*)에 감염된 인간의 위 상피 세포주인 AGS 세포주에서 COX-2와 세포유착분자인 integrin 발현이 증가함이 보고되었다. 이번 연구에서는 *H. pylori*에 의해 PAR-2 발현이 유도 되는지 또한 *H. pylori* 감염에 의한 COX-2와 integrin의 증가가 PAR-2와 관련이 있는지 알아보고자 한다. *H. pylori*를 AGS 세포주에 100:1의 비율로 처치하고 PAR-2와 integrin의 mRNA와 단백 발현을 역전사중합연쇄반응과 immunoblotting을 통해서 확인하였다. 또한 integrin의 어떤 소단위가 *H. pylori*에 의해 증가한 세포 유착에 관여하는지 adhesion assay로 확인하였다.

연구 결과 *H. pylori*로 자극한 AGS 세포주에서 PAR-2가 활성화되고 integrin $\alpha_5\beta_1$이 유도되었다. *H. pylori*는 AGS 세포주의 세포유착을 integrin $\alpha_5\beta_1$을 통해 증가시켰다. *H. pylori*에 의해 증가한 COX-2와 integrin $\alpha_5\beta_1$ 발현은 PAR-2 antisense oligodeoxynucleotide(AS ODN)에 의해 억제되었다. 이는 *H. pylori*에 감염된 세포주의 COX-2와 integrin $\alpha_5\beta_1$ 발현 증가는 PAR-2에 의해 매개됨을 시사한다.

*H. pylori*는 세포외기질 단백들(poly-L-lysine, fibronectin, collagen, laminin)과

integrin $\alpha_5\beta_1$ 과 붙어 세포유착을 증가시키나 G-단백 억제제인 pertussis toxin과 Src 인산화 억제제인 herbimycin A에 의해 세포유착이 유의하게 감소되었다. 이러한 결과로 *H. pylori*는 PAR-2/G-단백 신호체계를 자극하여 세포유착을 조절한다고 생각한다.

이상의 결과들로 종합하여 볼때 *H. pylori*는 인간의 위상피 세포주의 COX-2와 integrin $\alpha_5\beta_1$을 증가시켜 세포유착을 유도하는데 이는 PAR-2 활성화에 의해 매개된다고 생각한다.

핵심되는 말: *Helicobacter-pylori*, cyclooxygenase-2, integrin $\alpha_5\beta_1$, proteinase-activated receptor-2