

Flagellin promotes the proliferation of gastric cancer cells via the Toll-like receptor 5

EUN-JUNG SONG¹, MIN-JUNG KANG¹, YONG-SEOK KIM², SUN-MOON KIM²,
SANG-EOK LEE³, CHANG-HWAN KIM⁴, DONG-JAE KIM⁵ and JONG-HWAN PARK¹

Departments of ¹Biochemistry, ²Internal Medicine and ³Surgery, College of Medicine, Konyang University, Daejeon 302-711; ⁴Agency for Defense Development, Daejeon 305-152, Republic of Korea; ⁵Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

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Abstract. Signaling of the Toll-like receptor (TLR) is closely associated with tumor development and progression processes including cell proliferation, angiogenesis, metastasis, and immunosuppression. In this study, we examined the expression of TLR5 in gastric cancer cells and its function in cell proliferation. RT-PCR revealed that the TLR5 gene was expressed in all gastric cancer cell lines examined, SNU638, SNU601, SNU216, and AGS. The TLR5 agonist, flagellin, induced IL-8 production and NF- κ B activation in the gastric cancer cell lines. In addition, flagellin enhanced the proliferation of all gastric cancer cells examined, whereas LPS did not affect that of SNU638 cells. Blockade of TLR5 using an antibody, restored the proliferation of SNU638 cells enhanced by flagellin, indicating that TLR5 is essential for cell proliferation by flagellin. Flagellin also led to phosphorylation of ERK in SNU638 cells. The ERK inhibitor, PD98059, restored the proliferation ability of SNU638 cells enhanced by flagellin, suggesting that ERK may play an important role in the proliferation of gastric cancer cells. These findings suggest that TLR5 may play an important role in tumor progression of gastric cancer via the regulation of cell proliferation.

Introduction

Pattern recognition receptors (PRRs) are a series of innate immune receptors including Toll-like receptors (TLRs), Nod-like receptors (NLRs), and an RNA helicase family of receptors. Sensing of pathogen-associated molecular patterns (PAMPs) by PRRs is critical for initiation of the inflammatory response. TLRs are type I transmembrane receptors which

are structurally characterized by extracellular leucine rich repeats (LRRs) and an intracellular Toll/IL-1R (TIR) domain. Extracellular surface TLRs including TLR2, TLR4, and TLR5 recognize bacterial lipoproteins, lipopolysaccharide (LPS), and flagellin, respectively (1). On the other hand, endosomal TLRs are composed of TLR3, TLR7/8, and TLR9 and are responsible for sensing nucleic acids such as dsRNA (TLR3), ssRNA (TLR7/8), and the CpG motif from bacterial and viral DNA (TLR9) (1). In addition to microbial molecules, a variety of endogenous ligands such as necrotic or dying cells, heat shock proteins, high mobility group box 1 (HMGB1), hyaluronan fragments, heparin sulphate, and fibronectin are recognized by TLR2 or TLR4 (2). TLR activation leads to the activation of NF- κ B or interferon regulatory factors (IRFs) through the MyD88-dependent and/or MyD88-independent (TRIF-dependent) pathway and subsequently produce pro-inflammatory cytokines or type I interferons (1,3).

TLRs are also known to be closely associated with tumor development (4,5). Many studies have shown that TLR activation promotes tumorigenesis in a variety of carcinomas by enhancing cell proliferation, angiogenesis, and immunosuppression (6-13). In addition, TLRs mediate migration or invasion of several tumors, which are critical processes for cancer metastasis (7,14-16).

Gastric adenocarcinoma is a leading cause of cancer-related death in the world, ranking as the second cause in males and the fourth in females (17). Over the world, more than 700,000 people per year die due to this malignancy (18). *Helicobacter pylori* infection is the best-known risk factor for gastric adenocarcinoma, and etiological studies have revealed that the attributable risk for gastric cancers conferred by *H. pylori* is approximately 75% (19). There is evidence supporting the relation between TLR4 polymorphism and the risk of gastric cancers (20,21). It has also been reported that *H. pylori* augments the growth of gastric cancers via TLR2 or TLR4 pathways (22,23). These findings suggest that TLR signaling may play an important role in the development and progression of gastric cancer. A clinical study has shown that TLR5 was expressed in tumor cells of all patients with gastric cancer, even though TLR4 expression was also detected in a considerable number of patients (24). Although TLR5 was expressed and responded to *H. pylori* flagellin in some gastric

Correspondence to: Dr Jong-Hwan Park, Department of Biochemistry, College of Medicine, Konyang University, 685 Gasuwon-dong, Seo-gu, Daejeon 302-711, Republic of Korea
E-mail: jonpark@konyang.ac.kr

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cancer cells (25), it is unknown whether and how TLR5 is involved in promoting the proliferation of gastric cancer cells. In this study, we examined the expression and function of TLR5 in various gastric cancer cells (SNU638, SNU601, SNU216, and AGS) and assessed whether bacterial flagellin enhances the growth of gastric adenocarcinoma.

Materials and methods

Cell culture and reagents. Gastric epithelial cell lines, SNU638, SNU601, SNU216, and AGS (human origin), were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were incubated in RPMI medium including 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin at 37°C containing 5% CO₂. Flagellin (*Salmonella typhimurium* origin) was purchased from Invivogen (San Diego, CA, USA). The extracellular-signal regulated kinase (ERK) inhibitor, PD98059, was purchased from Selleck Chemicals (Houston, TX, USA). Isotype IgG and anti-TLR5 antibody were purchased from Imgenex (San Diego, CA, USA).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from each cell using easy-BLUE (Intron Biotechnology, Daejeon, Korea). Total RNA (1 µg) was reverse transcribed into cDNA and PCR was performed using the Power cDNA Synthesis kit (Intron Biotechnology) and the One-step RT-PCR AccuPower® HotStart PCR PreMix (Bioneer, Daejeon, Korea). The following primer sets were used: human TLR5, F: 5'-TGGGGGAACCTTACAGTTCG-3' and R: 5'-CTGGGATTCTCTGAAGGGG-3'; human GAPDH, F: 5'-CGGAGTCAACGGATTGTGTC-3' and R: 5'-AGCCTTCTCCATGGTGGTGA-3'.

Enzyme-linked immunosorbent assay (ELISA). The cells were treated with the indicated doses of flagellin for 24 h and the culture supernatant was collected. The concentration of IL-8 in culture supernatants was determined by a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA).

Western blot analysis. The cells were lysed in a buffer containing 1% Nonidet P-40 supplemented with a complete protease inhibitor cocktail (Roche, Mannheim, Germany) and 2 mM dithiothreitol. Lysates were resolved by 10% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane, and immunoblotted with primary antibodies against regular and phospho-IκB-α, p38, ERK, JNK, (Cell Signaling, Beverly, MA, USA). After immunoblotting with secondary antibodies, proteins were detected with an enhanced chemiluminescence (ECL) reagent (Intron Biotechnology).

Cell counting kit-8 (CCK-8) cell proliferation assay. The cells were treated with the indicated doses of flagellin for 3-4 days. Cell proliferation was determined by CCK-8 (Dojindo, Tokyo, Japan).

Statistical analysis. The differences between the mean values among the different groups were statistically assessed, and the values were expressed as the means ± SD. All of the statistical calculations were carried out using Microsoft Excel. Values of P<0.05 were considered significant.

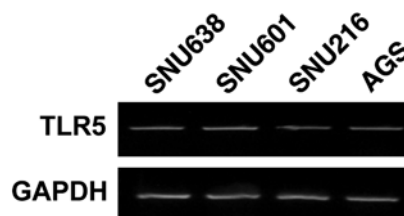


Figure 1. Gene expression of TLR5 in gastric cancer cells. RT-PCR was performed to examine TLR5 expression in four gastric cancer cell lines, SNU638, SNU601, SNU216, and AGS.

Results

Gene expression of TLR5 in gastric cancer cells. The gene expression of TLR5 was examined in the gastric cancer cell lines, SNU638, SNU601, SNU216, and AGS by RT-PCR. TLR5 mRNA was detectable in all tested cell lines and the expression level was similar between the cell lines (Fig. 1).

Flagellin leads to IL-8 production and NF-κB activation in gastric cancer cells. To determine whether TLR5 is functional in gastric cancer cells, the cells were treated with various doses of flagellin for 24 h and IL-8 production and NF-κB activation were examined. A large amount of IL-8 was produced in intact SNU638 cells, which was dose-dependently increased by flagellin (Fig. 2A). Likewise, treatment of flagellin increased IL-8 production in SNU601, SNU216, and AGS cells in a dose-dependent manner (Fig. 2B and C). However, LPS (a TLR4 agonist) did not affect IL-8 production in all cell lines (Fig. 2A and data not shown). Because the production of proinflammatory cytokines and chemokines is regulated by NF-κB, we examined the degradation and phosphorylation of IκB-α using Western blot analysis. Results showed that flagellin led to the phosphorylation of IκB-α 15 min after treatment in SNU638 and SNU601 cells (Fig. 2D and E). The degradation of IκB-α by flagellin was also shown 30 (in SNU638) or 60 min (in SNU601) after treatment (Fig. 2D and E). These findings indicate that TLR5 responds to its ligands and may play a role in cellular signaling in gastric adenocarcinoma.

Flagellin augments the growth of gastric cancer cells. It was examined whether flagellin affects the growth of gastric cancer cells using CCK-8. As shown in Fig. 3A, treatment with flagellin, but not LPS, enhanced the proliferation of SNU638 cells. Similarly, the growth of SNU601, SNU216, and AGS cells was also augmented by flagellin (Fig. 3B). When TLR5 was blocked by its antibody, flagellin did not enhance the proliferation of SNU638 cells (Fig. 3C). These findings suggest that TLR5 may be involved in regulating the growth of gastric adenocarcinoma.

Flagellin leads to phosphorylation of ERK in SNU638 cells. Mitogen-activated protein kinases (MAPKs) are known to mediate multi-cellular processes such as proliferation, differentiation, migration, and apoptosis (26,27). Western blot analysis was performed to determine whether flagellin induces the activation of MAPKs in gastric cancer cells. The results demonstrate that TLR5 stimulation with flagellin

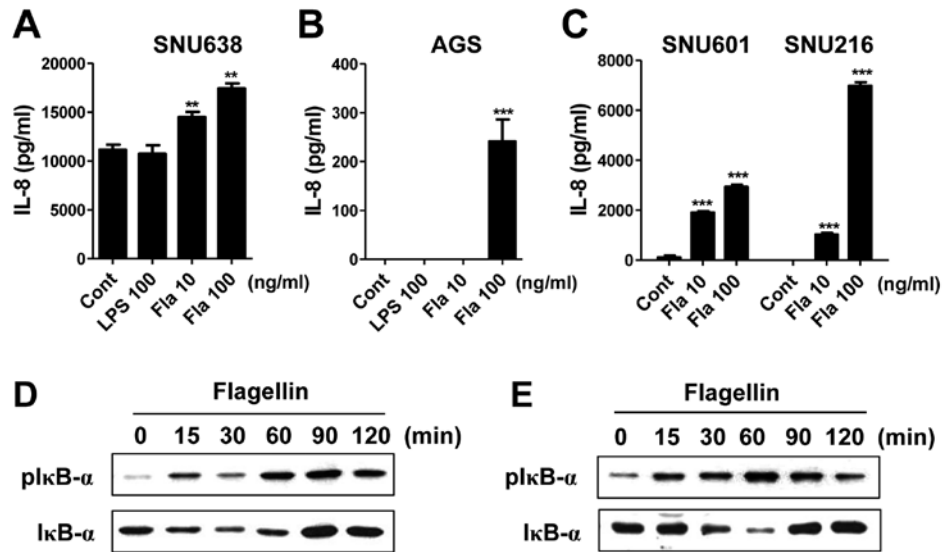


Figure 2. IL-8 production and NF- κ B activation in gastric cancer cells. The cells were treated with the indicated doses of flagellin or LPS for 24 h and IL-8 production was measured in the culture supernatant (A-C). SNU638 (D) and SNU601 (E) cells were treated with flagellin (100 ng/ml) and protein was extracted at the indicated time points. Immunoblotting was performed using antibodies for the regular and phosphorylated form of I κ B- α (D and E). **P<0.01, ***P<0.001.

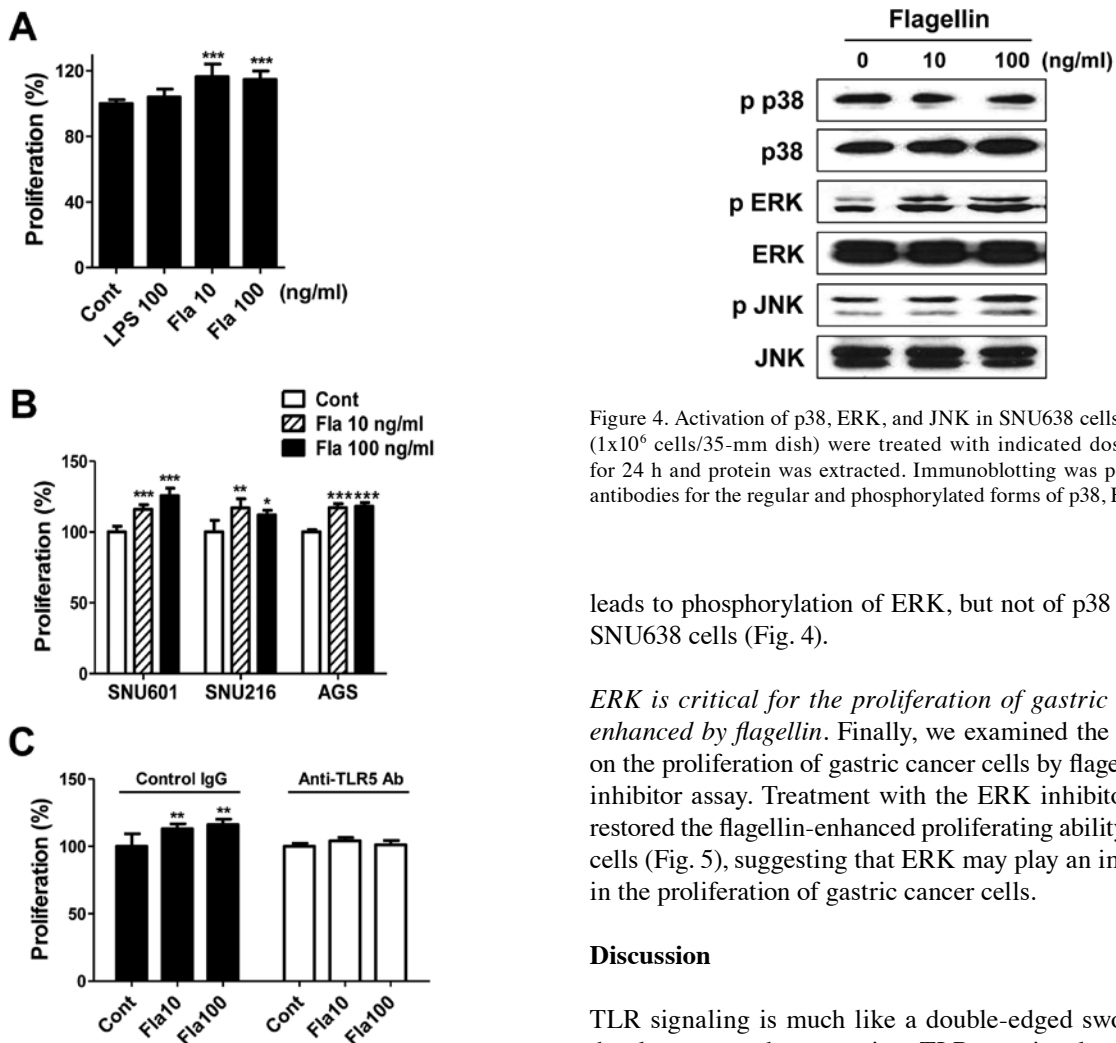


Figure 3. Cell proliferation assay. The cells (2×10^3 cells/well in a 96-well plate) were incubated with indicated doses of flagellin or LPS for 3-4 days and cell proliferation was examined by CCK-8 (A-C). Anti-TLR5 Ab and isotype control IgG were pretreated 2 h before flagellin treatment (C). *P<0.05, **P<0.01, ***P<0.001.

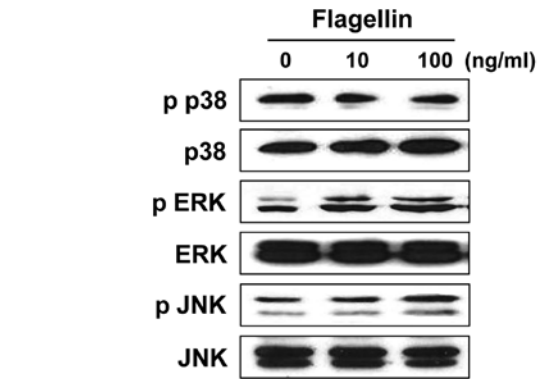


Figure 4. Activation of p38, ERK, and JNK in SNU638 cells. SNU638 cells (1×10^6 cells/35-mm dish) were treated with indicated doses of flagellin for 24 h and protein was extracted. Immunoblotting was performed using antibodies for the regular and phosphorylated forms of p38, ERK, and JNK.

leads to phosphorylation of ERK, but not of p38 and JNK, in SNU638 cells (Fig. 4).

ERK is critical for the proliferation of gastric cancer cells enhanced by flagellin. Finally, we examined the role of ERK on the proliferation of gastric cancer cells by flagellin using an inhibitor assay. Treatment with the ERK inhibitor, PD98059, restored the flagellin-enhanced proliferating ability of SNU638 cells (Fig. 5), suggesting that ERK may play an important role in the proliferation of gastric cancer cells.

Discussion

TLR signaling is much like a double-edged sword in tumor development and progression. TLRs are involved in not only tumor progression by promoting cell proliferation, migration, invasion, and angiogenesis, but also in tumor suppression by inducing cell death or immune activation (4,28), which are critically dependent on the tumor origin or the TLR type.

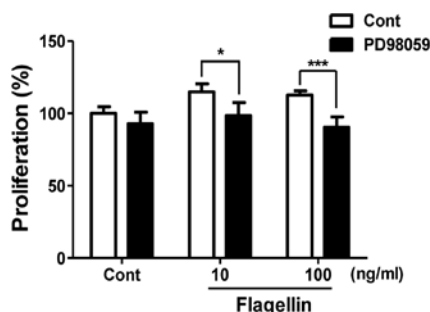


Figure 5. ERK inhibitor assay. SNU638 cells were treated with the ERK inhibitor, PD98059, for 2 h before flagellin treatment. The cell proliferation assay was performed as described in Fig. 3. * $P < 0.05$, *** $P < 0.001$.

The promotion of cell proliferation by microbial molecules via TLR signaling has been widely studied. Peptidoglycan, lipoteichoic acid, synthetic lipoprotein, and flagellin have been reported to increase epithelial cell growth via TLR2 or TLR5 (15). *Listeria monocytogenes* was shown to induce NF- κ B and MAPK activation and to promote the growth of H22 cells (a hepatocarcinoma cell line) via TLR2 signaling (22). TLR4 and TLR9 have been associated with cell proliferation of prostate epithelial cells through unknown mechanisms (29). These studies suggest that various TLRs are involved in tumor growth through distinct mechanisms.

In gastric cancers, TLR2 and TLR4 are known to promote cell proliferation (23,30). However, a recent clinical study revealed that TLR5 was strongly expressed in tumor cells of all examined patients ($n=22$) with gastric carcinoma (24), which drove us to examine the role of TLR5 on the growth of gastric cancer. In this study, we showed that the TLR5 gene was functionally expressed in all gastric cancer cell lines tested, which was supported by the enhanced IL-8 production and NF- κ B activation induced by a TLR5 agonist, flagellin. However, LPS did not increase IL-8 production in any of the cell lines examined, consistent with previous studies. Smith *et al* (25) revealed that two gastric epithelial cell lines, AGS and MKN45, express TLR4, but not MD-2 (a TLR4 co-receptor), which is essential for TLR4 function. A transfection assay also showed that LPS did not induce NF- κ B activation in MKN45 cells, suggesting that TLR4 is non-functional in these cells (25). Only TLR2 and TLR5, but not TLR4, were required for *H. pylori*-induced NF- κ B activation in MKN45 cells (25). In addition, it was clarified that *E. coli* LPS produced very low levels of IL-8 in intact gastric epithelial cells (MKN28 and MKN45 cells), which was due to a low level expression of TLR4 (30,31). However, *H. pylori* LPS up-regulated TLR4 expression in those cells via TLR2 signaling, which enhanced the *E. coli* LPS-induced IL-8 production (30). These findings suggest that TLR4 may be functional after stimulation increasing its expression in gastric epithelial cells, although it exhibits low activity under intact conditions.

In this study, flagellin enhanced the proliferation of all gastric cell lines tested, whereas LPS did not enhance the proliferation of SNU638 cells. This finding is in discordance with a study by Chochi *et al* (23) showing that treatment of LPS from *E. coli* and *H. pylori* up-regulated the proliferation of gastric cancer cell lines including MKN45, which was

mediated by TLR4. Although it can be due to differences in the experimental protocol and the conditions, the exact reason for the discrepancy remains to be elucidated.

ERK is associated with tumor proliferation and malignancy in gastric cancers. *H. pylori* LPS induced the proliferation of gastric cancer cells via the MEK1/2-ERK1/2 pathway (30). TGF- β also promoted invasion and metastasis of gastric cancer cells via the ERK and JNK signaling pathway (32). In addition, *Mycoplasma hyorhinis* infection promoted tumor cell migration, invasion, and metastasis, which was possibly associated with the enhanced ERK phosphorylation (33). In this study, flagellin enhanced ERK phosphorylation in SNU638 cells and the ERK inhibitor, PD98059, restored the proliferating ability of SNU638 cells enhanced by flagellin, suggesting that flagellin may promote cell proliferation through an ERK-dependent pathway.

In conclusion, our study revealed that TLR5 activation enhances the proliferation of gastric cancer cells through the ERK signaling pathway. Further studies examining TLR5 functions, such as angiogenesis, metastasis, and immune modulation in gastric cancers and its physiological role using an *in vivo* tumor model are warranted.

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