Uncontrolled Inflammation Induced by AEG-1 Promotes Gastric Cancer and Poor Prognosis

Guanghua Li, Zhao Wang, Jinning Ye, Xinhua Zhang, Hui Wu, Jianjun Peng, Wu Song, Chuangqi Chen, Shirong Cai, Yulong He, and Jianbo Xu

Abstract

Gastric cancer is one of the most common causes of cancer-related death worldwide. Helicobacter pylori infection plays an important role in the development and progression of gastric cancer. The expression of astrocyte-elevated gene-1 (AEG-1) is increased in gastric cancer tissues, thereby contributing to the inflammatory response. We investigated whether and how AEG-1 regulated proinflammatory signaling in gastric cancer cells. We used human gastric cancer cell lines and athymic nude mice to investigate the role of AEG-1 in the regulation of the TLR4/nuclear factor-κB (NF-κB) signaling pathway and cancer invasion and compared the expression of AEG-1 and related proteins in 93 patients with gastric cancer by immunohistochemistry. In human gastric cancer cells, both AEG-1 and TLR4 could be induced by lipopolysaccharide (LPS) stimulation. AEG-1 was upregulated via LPS-TLR4 signaling and in turn promoted nuclear translocation of the NF-κB p65 subunit. At the same time, AEG-1 overexpression decreased the levels of suppressor of cytokine signaling (SOCS) protein SOCS-1, a negative regulator of the TLR4 pathway. Furthermore, nude mice engrafted with AEG-1/TLR4-expressing cells demonstrated larger tumor volumes than control animals. In patients with gastric cancer, the expression of AEG-1 correlated with that of TLR4, SOCS-1, and NF-κB and was higher in tumors compared with noncancerous adjacent tissues. Overall survival in patients with gastric cancer with simultaneous expression of AEG-1 and TLR4 was poor. Our results demonstrate that AEG-1 can promote gastric cancer progression by a positive feedback TLR4/NF-κB signaling-related mechanism, thus providing new mechanistic explanation for the role of inflammation in cancer progression. Cancer Res; 74(19): 5541–52. ©2014 AACR

Introduction

Gastric cancer remains the fourth most common cancer and the second leading cause of cancer-related death in the world (1). Early detection, dietary changes, and reduction in chronic Helicobacter pylori infection have resulted in a substantial decrease of stomach cancer rates in most parts of the world (2, 3). However, China alone accounts for nearly 42% of all gastric cancer cases worldwide (4).

AEG-1, also known as metadherin (MTDH) or LYRIC, is induced in primary human fetal astrocytes infected with HIV-1 or treated with recombinant HIV-1 envelope glycoprotein (gp120) or tumor necrosis factor-α (TNFα; refs. 5 and 6). AEG-1 is markedly overexpressed in many tumors such as breast cancer, hepatocellular carcinoma, and neuroblastoma (7–9). In our previous study, we have also observed high expression of AEG-1 in gastric cancer (10). Functional studies have demonstrated that AEG-1 can promote tumor proliferation, invasion, chemoresistance, angiogenesis, and metastasis (11–14).

AEG-1 oncogenic properties are linked to several signaling pathways, including those of nuclear factor-κB (NF-κB), PI3K/AKT, mitogen-activated protein kinase (MAPK), and Wnt (15). The NF-κB pathway, which plays an important role in inflammation and tumorigenesis, was the first one shown to be activated by AEG-1 (16). It is well known that inflammatory responses play decisive roles at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis (17). H. pylori can either directly promote gastrointestinal carcinogenesis through the enhanced production of free radicals or indirectly through the establishment of carcinogenic environment because of long-term inflammation of the gastric mucosa, which eventually results in mucosal atrophy, intestinal metaplasia, and cancer. Accordingly, chronic inflammation caused by H. pylori is a major step in the initiation and development of gastric cancer (18). However, the exact mechanism remains unclear.

Our previous study has demonstrated that AEG-1 expression is upregulated in gastric cell lines and clinical specimens and is associated with unfavorable prognosis (10). We also observed that patients with gastric cancer with H. pylori infection had poor overall and relapse-free survival (19). H. pylori is a gram-negative bacterium characterized as a class I carcinogen for its...
role in the pathogenesis of gastric cancer (20). Lipopolysaccharide (LPS) is the most important component of the outer membrane of gram-negative bacteria and a TLR4 ligand, which has been shown to induce AEG-1 expression in human monocytes (21) and breast cancer cells (22).

In this study, we explored the role of AEG-1 in the regulation of TLR4/NF-kB signaling in gastric cancer. Our results indicate that AEG-1 directly regulates the expression of NF-kB-dependent genes in cancer cells. As expected, AEG-1 could be induced by LPS stimulation in TLR4-positive gastric cancer cells. Furthermore, we also present evidence of a positive feedback loop between AEG-1 and the TLR4/NF-kB signaling pathways. This study shows that AEG-1 plays a role in gastric cancer progression via inflammatory mechanisms, suggesting that it could be a potential target for therapeutic intervention in gastric cancer.

Patients and Methods

Tissue samples, cell culture, and chemicals

Patients with gastric cancer who underwent curative surgery in the Gastrointestinal Surgery Department of the First Affiliated Hospital of Sun Yat-sen University from 2006 to 2007 were included in this study. There were 64 men and 29 women between 24 and 82 years old (average age, 56.3 years). Informed consent was obtained from all patients. Four samples of gastric cancer tumors and the matched adjacent noncancerous gastric tissues were obtained by biopsy and frozen at −80°C.

Gastric cancer cell lines SGC7901, AGS, HGC27, MGC803, and BGC823 and normal gastric epithelial GES-1 cells were obtained from the type Culture Collection of Chinese Academy of Sciences (Shanghai, China). These cell lines were authenticated using short tandem repeat (STR) profiling, isoenzyme analysis, and cell viability analysis. All cell lines used in this study were regularly screened for mycoplasma contamination and tested negative for the same. AGS cells were cultured in F12 medium, and other cells were grown in RPMI-1640 medium (both media supplemented with 10% fetal bovine serum (FBS)), 50 U/mL penicillin, and 50 mg/mL streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Escherichia coli LPS (0111:b4) and proteasome inhibitor MG132 were purchased from Sigma Chemical Co.

Cell transfection with plasmids and siRNAs

The expression plasmids for AEG-1 (EX-T7038-M02), TLR4 (EX-K2201-M02), and SOCS-1 (EX-K2201-M02), and the GFP-expressing vector M02 were purchased from GeneCopoeia. All siRNAs were obtained from RiboBio. The primers used were as follows: si-AEG-1 sense, 5'-CAGAAUAATAACAGUCAAdTdTT-3' and antisense, 5'-dTdTGCUGUAAUUGGAGACGUU-3'; si-TLR-4 sense, 5'-GGUGUGAAACAGACAGAAUdTdT-3' and antisense, 5'-dTdTCCACAUUUUGGUCGUU-3'. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Immunohistochemistry and immunofluorescence

Immunohistochemistry analysis of formalin-fixed and paraffin-embedded gastric tissues was performed as previously described (10) using primary antibodies against AEG-1/MTDH (1:100; Epitomics), TLR4, NF-kB (both 1:50; Abgent), and SOCS-1 (1:50; Sigma-Aldrich).

The immunostaining index was based on the proportion of positively stained tumor cells and staining intensity. The proportion of positively stained tumor cells was graded as 0 (no positively stained cells), 1 (<10%), 2 (10%–50%), and 3 (>50% of positive cells), and staining intensity was scored as 0 (no staining), 1 (light yellow), 2 (yellow brown), and 3 (brownish-yellow staining). The immunostaining index was then calculated as staining intensity score multiplied by the proportion of positively stained tumor cells; tumors with the indexes 0 to 2 were considered immunostaining-negative and those with 3 to 9 were scored immunostaining-positive.

Cells cultured in 24-well plates were transfected with either 50 or 100 nmol/L scrambled siRNA and si-AEG-1 for 48 hours. Cells were fixed with 4% formaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS for 30 minutes, rinsed with PBS, and incubated with NF-kB antibody at 4°C overnight and then with Alexa Fluor 488–conjugated anti-rabbit IgG (Invitrogen) for 1 hour at room temperature. DAPI (KGA215; KeyGen Biotech) was used to stain the nuclei. Images were analyzed using an Olympus fluorescence microscope.

Quantitative real-time RT-PCR

Total RNA was extracted from cells or tissues with RNAiso (Takara) following the manufacturer’s protocol, and 500 ng was reverse-transcribed using PrimeScript RT Master Mix (Takara). Quantitative real-time RT-PCR (qRT-PCR) was performed with cDNA as a template in the presence of SYBR Premix Ex Taq (Tli RNaseH Plus; Takara). The primers are listed in Supplementary Table S1. The levels of mRNA were normalized to that of GAPDH.

Protein extraction and immunoblotting

Whole-cell lysates were prepared using the Whole Cell Protein Extraction Kit; nuclear and cytoplasmic proteins were isolated using the Nuclear and Cytoplasmic Protein Extraction Kit (KeyGen Biotech). Protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Equal amounts of protein were resolved by SDS-PAGE using 10% or 12% gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in PBS and probed with primary antibodies specific to the NF-kB subunit p65 (1:1,000; Cell Signaling Technology), AEG-1 (1:1,000), lamin B (1:1,000; Epitomics), TLR4 (1:1,000), and SOCS-1 (1:1,000). Protein expression levels were normalized to that of GAPDH.

NF-kB–dependent luciferase assay

Gastric cancer cells transfected with AEG-1 siRNA or scrambled siRNA were incubated for 48 hours in a 48-well plate and transfected with 0.2 μg/well of the pGL4.32[luc2P/NF-kB-RE/Hygro] vector and an equal amount of the pRL-TK plasmid (Promega) using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s recommendations. After 48 hours of incubation, luminescent signals were measured using the Dual-Luciferase Reporter Assay Kit (Promega) according to
the manufacturer’s protocol. The data are presented as the mean ± SD of three independent experiments.

Invasion assay

Cell invasion was assessed in 24-well BioCoat Invasion chambers containing membranes coated with Matrigel (BD Biosciences), according to the manufacturer’s instructions. Cells (5 × 10^5/well) were transfected with the vector (V), V-AEG-1, V-TLR4, or V-AEG-1 and V-TLR4 were seeded in the upper chambers in 1% FBS-containing RPMI-1640, whereas the lower chambers were filled with RPMI-1640 supplemented with 5% FBS. Cells were incubated at 37°C for 24 hours; the membranes were removed, fixed, and stained with crystal violet. Cells on the upper membrane surface were removed by wiping with a cotton swab and invasion was determined by counting the cells migrated to the lower membrane side under the microscope. At least 5 fields were counted in each experiment performed in triplicate.

Cell-proliferation assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and colony-formation assay were used to assess cell proliferation. For the MTT assay, cells transfected with the vector (V), V-AEG-1, V-TLR4, or V-AEG-1, and V-TLR4 were seeded in 96-well plates (5 × 10^4 cells/well) in medium containing 10% or 1% FBS with or without LPS and cultured for 24, 48, and 72 hours. For the colony-formation assay, cells were seeded in 60-mm plates (200 cells/plate) in RPMI-1640 and treated with the indicated pharmacologic inhibitors or LPS. Colonies of ≥50 cells were scored after 2 weeks.

Xenograft studies in athymic nude mice

Cancer cells transfected the vector (V), V-AEG-1, V-TLR4, or V-AEG-1 and V-TLR4 were established as subcutaneous xenografts in the flanks of 5-week-old male Balb/c nu/nu mice (Guangdong Medical Laboratory Animal Center, China) using 5 × 10^6 cells of each cell line (5 mice/line). Tumor volumes were measured every 3 days with a caliper and calculated using the following formula: larger diameter × (smaller diameter)^2/2.

Statistical analysis

Data were analyzed using the SPSS 15.0 statistical software. The results are expressed as the mean ± SD of at least three independent experiments. The difference between groups was assessed by the unpaired two-tailed Student t test. A P value of <0.05 was considered statistically significant.

Results

Upregulation of AEG-1 and TLR4 in gastric cancer tissues and cancer cell lines

RT-PCR and Western blotting analyses were used to investigate mRNA and protein expression of AEG-1 and TLR4 in 4 patients (paired gastric tumor tissues and matched normal mucosal) and in human gastric cell lines (SGC7901, AGS, HGC27, MGC803, and BGC823).

For all the 4 patients examined, protein and mRNA expression of both AEG-1 and TLR4 was higher in gastric tumors than in the adjacent noncancerous gastric tissues from the same patient (Fig. 1A–C). The expression was also higher in SGC7901, MGC803, and BGC823 gastric cancer cells than in normal gastric epithelial GES-1 cells (Fig. 1D–F). Because the SGC7901 cell line expressed the highest levels of TLR4, it was chosen for the investigation of the TLR4 signaling pathway.

LPS induces AEG-1 expression in gastric cancer cells

To investigate whether the TLR4 signaling pathway was involved in AEG-1 upregulation by LPS, we knocked down TLR4 expression by siRNA. The efficiency of TLR4 downregulation by 3 TLR4-specific siRNAs was tested by RT-PCR and immunoblotting (Fig. 2A and B). The effect of LPS on AEG-1 protein expression in gastric cancer cells was examined by immunoblotting. When cells were stimulated with 100 ng/mL of LPS for 0.5, 1, 2, and 3 hours, AEG-1 levels showed a time-dependent increase up to 1 hour (Fig. 2C). When various LPS concentrations were tested, AEG-1 was upregulated in a concentration-dependent manner up to 100 ng/mL (Fig. 2D). Of the 3 specific siRNAs, si-TLR4-2 was the most efficient in downregulating TLR4 expression, which in turn significantly reduced the expression of AEG-1 in response to LPS induction (Fig. 2E).

Next, we investigated the involvement of different cell signaling pathways in the regulation of the LPS-induced AEG-1 expression. The effect of a series of pharmacologic inhibitors, including those for mitogen-activated kinases p38 (SB203580), SAPK/JNK (SP600125), and MEK1/2 (U0126), and NF-κB (PDTC), on the LPS-induced AEG-1 expression was examined (Fig. 2F). Of all the inhibitors tested, only PDTC could prevent the induction of AEG-1 expression by LPS.

AEG-1 augments TLR4 expression and activates NF-κB

Next, we investigated time- and concentration-dependent effects of LPS on TLR4 expression in gastric cancer cells. When TLR4 expression was examined 0.5, 1, 2, and 3 hours after stimulation with 100 ng/mL LPS, it showed maximal increase at 1 hour (Fig. 3A, left). Similar to AEG-1, TLR4 expression was also upregulated by LPS in a concentration-dependent manner up to 100 ng/mL (Fig. 3A, right).

To investigate the role of AEG-1 in the TLR4 signaling pathway, we knocked down AEG-1 expression with 3 specific siRNAs (Fig. 3B). AEG-1 downregulation by the most efficient siAEG-1-3 resulted in a significant reduction of TLR4 response to LPS stimulation (Fig. 3C).

Previous studies revealed that AEG-1 could activate the NF-κB signaling pathway (16, 21, 23). Given that AEG-1 expression in gastric cancer cells was induced by LPS (Fig. 2C–E), we next tested whether AEG-1 regulated NF-κB activation in gastric cancer cells. NF-κB–dependent luciferase activity was markedly decreased in the cells transfected with AEG-1–specific siRNA (Fig. 3D), indicating that NF-κB signaling depended on AEG-1 expression.

In addition, AEG-1 downregulation negatively affected the nuclear translocation of NF-κB (Fig. 3E). A strong nuclear signal for the NF-κB subunit p65 observed in control (scrambled siRNA-transfected) cells, was significantly decreased in AEG-1 siRNA-expressing cells with a corresponding increase in the cytoplasmic staining (Fig. 3E, left). The cytoplasmic localization...
of p65 following AEG-1 knockdown was also confirmed by analyzing p65 levels in cytoplasmic and nuclear extracts by immunoblotting (Fig. 3E, right). The levels of p65 protein increased in the cytoplasm and decreased in the nuclei of gastric cancer cells after AEG-1 knockdown, indicating that AEG-1 negatively regulated NF-κB activation in gastric cancer cells.

Next, we evaluated the expression of NF-κB downstream genes IL6, IL8, matrix metalloproteinase-2 (MMP2), MMP9, and VEGF following AEG-1 knockdown. A significant decrease in IL8 and MMP9 mRNA levels was detected in gastric cancer cells transfected with si-AEG-1 compared with those transfected with scrambled siRNA (Fig. 3F).

AEG-1 regulates NF-κB signaling through a positive feedback mechanism involving downregulation of SOCS-1
Suppressor of cytokine signaling (SOCS) proteins SOCS-1 and SOCS-3 are believed to play pivotal roles in inflammation and in the development and progression of cancer. Previous studies have shown that SOCS-1 and SOCS-3 can inhibit TLR4/NF-κB signaling in a negative feedback loop pattern (24–26). Upon translocation to the nucleus, SOCS-1 ubiquitin ligase interacts with NF-κB subunit p65 and promotes its proteasomal degradation (27), thus terminating the expression of NFκB-inducible genes. On the other hand, nuclear-translocated AEG-1 causes transcriptional activation of NF-κB target genes (16). We hypothesized that in gastric cancer cells, AEG-1 regulated TLR4/NF-κB signaling via SOCS proteins. To explore this possibility, we examined SOCS-1 and SOCS-3 expression in gastric cancer cells transfected with the V-AEG-1 plasmid. The transfection efficiency exceeded 80% (Fig. 4A), and the expression of AEG-1 was confirmed by RT-PCR and immunoblotting (Fig. 4B). AEG-1 expression affected the level of SOCS-1 but not that of SOCS-3 (data not shown). The effect of AEG-1 on SOCS-1 expression was further investigated in the gastric cancer cells cotransfected with V-AEG-1 and V-SOCS-1 plasmids. Western blot analysis showed that SOCS-1 protein levels were significantly reduced in AEG-1–transfected cells, in contrast to robust SOCS-1 expression observed in control MO2–transfected cells (Fig. 4D). Given that the AEG-1 and MO2 constructs were based on the same plasmid, the effect on SOCS-1 expression was AEG-1 specific.

We suggested that AEG-1 overexpression promoted the proteasomal degradation of SOCS-1. This hypothesis was tested by treating the transfected gastric cancer cells for 48 hours with MG-132, a cell-permeable proteasome inhibitor. The proteasomal inhibitor restored SOCS-1 protein expression despite the presence of AEG-1 (Fig. 4E), suggesting that AEG-1–promoted proteasomal degradation of the SOCS-1 protein.

AEG-1 and TLR4 coexpression promote malignancy of gastric cancer cells
Gastric cancer cells transfected with the AEG-1 or TLR4 expression plasmids or both were selected for stable
expression with G418. The analysis of cell proliferation by the MTT assay revealed that compared with control cells (vector), AEG-1- or/and TLR4—expressing cells had higher proliferation (Fig. 5A) and colony-formation (Fig. 5B) rates. In the Matrigel invasion assay, gastric cancer cells cotransfected with AEG-1 and TLR4 showed a markedly upregulated invasive ability compared with that of other groups (Fig. 5C). Establishment of subcutaneous xenograft in the flanks of nude mice revealed that AEG-1/TLR4 coexpressing cancer cells generated large aggressive tumors in 4 weeks (Fig. 5D).

Increased expression of AEG-1 and TLR4 indicates poor prognosis for gastric cancer

The expression of AEG-1, TLR4, SOCS-1, and NF-κB was analyzed in the formalin-fixed and paraffin-embedded gastric mucosa tissues by immunohistochemistry. Compared with the matched normal gastric tissues, tumors showed high protein expression of AEG-1, TLR4, and NF-κB, but not SOCS-1 (Fig. 6A). In the adjacent noncancerous tissues, expression of AEG-1, TLR4, SOCS-1, and p65 was detected in 3 (15%), 5 (25%), 18 (90%), and 7 (35%) of 20 patients, respectively, whereas in gastric tumors, expression of these proteins were observed in 63 (67.7%), 61 (65.6%), 45 (48.4%), and 66 (70.9%) of 93 patients (P < 0.001, P = 0.001, P = 0.01, and P = 0.02, respectively). The association between AEG-1, TLR4, SOCS-1, and p65 expression and clinicopathologic features is shown in Table 1. A significant correlation was detected between AEG-1 expression and the depth of tumor invasion and UICC stage. A significant difference in histologic differentiation and UICC stage was observed between the groups positive and negative for TLR4 protein expression. Significant differences in tumor size, depth of tumor invasion, lymph node metastasis, distant metastasis, and UICC stage were detected between the patients.
positive and negative for SOCS-1 expression, whereas tumor location and the depth of tumor invasion were associated with p65 levels. However, no correlation was observed between the expression of these proteins and sex, age, adjuvant treatment, invasion of adjacent organs, surgery, or carcinoembryonic antigen (CEA; data not shown).

We also analyzed the correlation between the levels of AEG-1 and those of TLR4, SOCS-1, and p65. Our data revealed that...
AEG-1 expression positively correlated with that of TLR4 and NF-κB but negatively with that of SOCS-1 (Supplementary Table S2).

We then investigated whether AEG-1 and TLR4 expression had an impact on the survival of patients with gastric cancer. Among 93 patients, the median overall survival time was 64.2 months. Overall survival was 46.0 months (95% CI, 38.2–53.8) in the patients positive for AEG-1 expression and 63.0 months (95% CI, 53.3–72.8) in the AEG-1–negative patients (P = 0.038; Fig. 6B). Similarly, overall survival for the patients positive and negative for TLR4 expression was 46.9 months (95% CI, 38.9–54.9) and 60.5 months (95% CI, 50.5–70.5; P = 0.033), respectively (Fig. 6C). We next assessed the effects of simultaneous expression of AEG-1 and TLR4 on overall survival. As expected, overall survival for the AEG-1/TLR4–positive patients was lower than that for AEG-1/TLR4–negative patients: 43.7 months (95% CI, 34.7–52.6) compared with 69.1 months (95% CI, 60.0–78.1; P = 0.033), respectively (Fig. 6D).

Discussion

A strong association between chronic infection, inflammation, and cancer has been revealed by clinical and epidemiologic studies (28–30). The link between inflammation and cellular malignant transformation was first suggested by Rudolf Virchow in the 19th century. Thus, a correlation has been shown between gastric cancer and H. pylori infection, colon cancer and inflammatory bowel disease, liver cancer and chronic viral hepatitis, and cervical cancer and HPV infection (28–30). These studies suggest that chronic inflammation plays an important role in tumor initiation and progression (30). An oncogenic protein AEG-1 is involved in cancer-related inflammation (31); however, the AEG-1–specific regulatory mechanisms are not well understood. Here, we show that LPS can upregulate the expression of AEG-1, which in turn augments the TLR4/NF-κB signaling pathway.

Although AEG-1 does not directly bind to DNA, upon TNFα treatment it interacts with the NF-κB p65 subunit and cyclic AMP-responsive element binding protein (CREB)–binding
protein (CBP) on the IL8 promoter, increasing IL8 transcription (32). IL8, an NF-κB downstream gene, positively regulates angiogenesis and metastasis, whereas inhibition of NF-κB abrogates AEG-1–induced augmentation of growth and invasion of HeLa cells (16). Gene chip analysis revealed that AEG-1 overexpression led to the upregulation of several NF-κB downstream genes, including cell adhesion factors ICAM-2 and ICAM-3; selectin E, L, and P ligands; many other important mediators of tumor malignancy, such as IL6, IL8, TLR4, and TLR5; and transcription factors c-Jun and c-Fos (16). Consistent with these data, our study also demonstrated that AEG-1 knockdown could lead to the inactivation of NF-κB and downregulation of IL8 and MMP9. AEG-1 knockdown also blocked LPS-induced TLR4 expression (Fig. 3), indicating the existence of a positive feedback loop between AEG-1 and the TLR4/NF-κB signaling pathway. Chromatin immunoprecipitation assays revealed that AEG-1 might bridge NF-κB, CBP, and basal transcription machinery by functioning as a coactivator in NF-κB–mediated transcription (32).

Toll-like receptors (TLR) play a key role in the innate immune system (33). TLR4 was the first discovered human TLR activated by LPS of gram-negative bacteria (34). TLR4 is expressed not only in immune cells but also in many tumors, including breast cancer (35) and colon and ovarian cancer (36) where TLR4-related signaling can promote tumor growth and immune escape (33, 37, 38). Here, we revealed strong association of TLR4 with gastric cancer by demonstrating higher TLR4 expression in gastric cancer cell lines and gastric tumors compared with normal gastric cells and noncancerous tissues (Fig. 1). TLR4 stimulation with bacterial LPS has been shown to promote tumor invasion and metastasis through NF-κB–dependent upregulation of inducible nitric oxide synthase (iNOS) and MMP2 (39). Moreover, TLR4 activation by LPS in tumor cells induces the synthesis of various proinflammatory soluble factors, including IL6, IL12, and iNOS, thus creating the inflammatory microenvironment and promoting tumor growth (38). Besides, H. pylori infection can actively induce inflammation by upregulating TLR4 expression in gastric epithelial cell lines, which in turn can be enhanced by other bacteria and/or endotoxins in the gastrointestinal tract (40, 41). Our results also indicate that TLR4 expression can be induced by LPS stimulation. After TLR4 knockdown, the LPS–induced AEG-1 expression diminished correspondingly and the NF-κB inhibitor PDTC had a similar effect, indicating that TLR4/NF-κB signaling can regulate AEG-1 expression (Fig. 2E and F).
SOCS proteins, originally identified as negative-feedback mediators of cytokine signaling, also play a key role in the negative regulation of TLR signaling. SOCS proteins, especially SOCS-1 and SOCS-3, are implicated in inflammation and in the development and progression of cancers (42, 43). Previous studies have shown that SOCS-1 and SOCS-3 inhibit TLR signaling through the adaptor protein Mal, TNF receptor–associated factors 3 and 6, and NF-κB (26, 44, 45). SOCS-1 is the only member of the SOCS family that can interact with the NF-κB p65 subunit, promoting its polyubiquitination and proteasomal degradation and inhibiting NF-κB signaling (27). On the other hand, AEG-1 interaction with p65 upregulates NF-κB pathway (16), although the mechanism remains unknown. AEG-1 may regulate NF-κB signaling either by binding to other coactivators such as CREB-binding protein (CBP; ref. 32) or by decreasing NF-κB inhibitory protein IκB. Our investigation showed that AEG-1 could downregulate SOCS-1 but not SOCS-3 (Fig. 4). Even though the function of SOCS-1 in tumors is still a debatable matter, SOCS-1 is considered an important tumor suppressor in many malignancies, including melanoma, breast, and prostate cancers (46). Here, we have demonstrated that AEG-1 regulation of SOCS-1 protein underlies low expression of SOCS-1 in tumors. In this context, AEG-1 can activate the TLR4/NF-κB pathway by downregulating SOCS-1, an inhibitor of the TLR4/NF-κB signaling. In addition, AEG-1 might reduce SOCS-1 levels via the ubiquitin–proteasome pathway (Fig. 4), thus providing a potential mechanism for the posttranslational regulation of SOCS-1 activity. AEG-1 affects mRNA processing and/or stability, which can also lead to decreased SOCS-1 protein levels. This hypothesis requires further investigation.

The long-term consequence of H. pylori infection is gastric cancer. The activation of NF-κB and upregulation of IL8 in gastric epithelial cells have been suggested as critical factors in the development of gastric cancer. SOCS proteins, especially SOCS-1 and SOCS-3, are known to inhibit NF-κB signaling by binding to the NF-κB p65 subunit and promoting its polyubiquitination and proteasomal degradation. Our study shows that AEG-1, a coactivator for NF-κB, can downregulate SOCS-1, thus activating the TLR4/NF-κB pathway. This finding suggests a potential mechanism for the posttranslational regulation of SOCS-1 activity and highlights the importance of AEG-1 in the development of gastric cancer.

Figure 6. Patients with gastric cancer positive for AEG-1 and TLR4 expression have poor prognosis. A, AEG-1, TLR4, SOCS-1, and NF-κB expression in gastric cancer and normal mucosal tissues (magnification, ×200). Survival curve for patients with cancer expressing AEG-1 (B), TLR4 (C), and AEG-1 with TLR4 (D).
Table 1. Clinicopathologic features of 93 patients with gastric cancer assessed for AEG-1, TLR4, SOCS-1, and NF-κB expression

<table>
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<th>AEG-1 positive (n = 63; 100%)</th>
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<th>TLR4 negative (n = 32; 100%)</th>
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<th>P</th>
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<td>&lt;4 cm</td>
<td>16 (53.3)</td>
<td>25 (39.7)</td>
<td>0.215</td>
<td>15 (46.9)</td>
<td>26 (42.6)</td>
<td>0.695</td>
<td>13 (27.1)</td>
<td>28 (62.2)</td>
<td>0.001</td>
<td>13 (48.1)</td>
<td>28 (42.4)</td>
<td>0.614</td>
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<td>≥4 cm</td>
<td>14 (46.7)</td>
<td>38 (60.3)</td>
<td>17 (53.1)</td>
<td>35 (57.4)</td>
<td>35 (72.9)</td>
<td>17 (37.8)</td>
<td>14 (51.9)</td>
<td>38 (57.6)</td>
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<td>9 (30.0)</td>
<td>20 (31.7)</td>
<td>0.865</td>
<td>9 (28.1)</td>
<td>20 (32.8)</td>
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<td>43 (68.3)</td>
<td>23 (71.9)</td>
<td>41 (67.2)</td>
<td>29 (60.4)</td>
<td>35 (77.8)</td>
<td>14 (51.9)</td>
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<td>21 (33.3)</td>
<td>0.349</td>
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<tr>
<td>T1/T2</td>
<td>18 (60.0)</td>
<td>17 (27.0)</td>
<td>0.002</td>
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<td>22 (36.1)</td>
<td>0.666</td>
<td>11 (22.9)</td>
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<td>T3/T4</td>
<td>12 (40.0)</td>
<td>46 (73.0)</td>
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<td>37 (77.1)</td>
<td>21 (46.7)</td>
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<td>8 (16.7)</td>
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mechanisms responsible for \textit{H. pylori}–induced chronic inflammation and gastric carcinogenesis (47). The expression of most cancer biomarkers is driven by NF-\kappaB–regulated genes (48). For example, cancer-secreted IL8 can enhance the proliferation and survival of tumor cells via autocrine signaling pathways, whereas tumor-derived IL8 can promote the invasion and metastasis of cancer cells and activate endothelial cells in the tumor vasculature to enhance angiogenesis (48, 49). MMP9 can be an important modulator of tumor progression by affecting processes critically involved in tumorigenesis and cancer progression, including growth, survival, angiogenesis, invasion, and regulation of the immune response (50). The activated AEG-1/NF-\kappaB feed-forward loop upregulates multiple cancer biomarkers and can significantly influence cancer progression. Our study demonstrates that AEG-1 potently activates NF-\kappaB signaling via regulation of TLR4 and SOCS-1 and that AEG-1 and TLR4 coexpression can greatly enhance the proliferation and malignancy of gastric cancer cells (Fig. 5). Furthermore, our investigation of 93 patients with gastric cancer reveals that the expression of AEG-1 correlates with that of TLR4, SOCS-1, and NF-\kappaB and together with that of TLR4, indicate poor prognosis and decreased survival (Fig. 6). Thus, AEG-1 can promote proinflammatory signaling via the AEG-1/NF-\kappaB feed-forward pathway and contribute to the initiation, progression, malignant conversion, invasion, and metastasis of gastric cancer (17).

In summary, we have demonstrated that in gastric cancer cells, AEG-1, induced by LPS/TLR4/NF-\kappaB signaling pathway, can in turn augment NF-\kappaB signaling by downregulating SOCS-1 and promoting uncontrolled inflammation in tumor microenvironment. AEG-1/NF-\kappaB can form a feed-forward loop to upregulate NF-\kappaB target genes in an autocrine manner, thus contributing to the progression of gastric cancer. Our results contribute to a deeper understanding of the association between AEG-1 and a sustained proinflammatory signaling in gastric cancer and could have a significant impact on both prevention and treatment of the disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Z. Wang, H. Wu, Y. He, J. Xu
Development of methodology: H. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Li, J. Ye, X. Zhang, H. Wu, C. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Peng, J. Xu
Writing, review, and/or revision of the manuscript: G. Li, J. Peng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zhang, W. Song, S. Cai, Y. He
Study supervision: W. Song, S. Cai, Y. He, J. Xu

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References


Uncontrolled Inflammation Induced by AEG-1 Promotes Gastric Cancer and Poor Prognosis

Guanghua Li, Zhao Wang, Jinning Ye, et al.


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