**Abstract.** Background: Chronic inflammation characterized by the recruitment and activation of macrophages has been implicated in the development of gastric cancer. Materials and Methods: Expression of the long form of pentraxin-3 (PTX3) in gastric cancer cells was examined by reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay. The migratory capacity of gastric cancer cells and chemotaxis of macrophages by PTX3 were assessed by wound-healing and transwell assays. PTX3 silencing using small interfering RNA (siRNA) was performed to confirm PTX3-mediated effects. Results: We demonstrated that PTX3 expression was elevated in human advanced gastric cancer tissues with increased infiltration of CD11b+ macrophages. Tumor necrosis factor-alpha increased PTX3 expression via nuclear factor-kappa B activation in human gastric cancer cells. PTX3 promoted the tumor cell migratory potential, the recruitment of macrophages and their subsequent binding to gastric cancer cells. These effects were suppressed by PTX3 knockdown using siRNA. Conclusion: Our findings suggest that gastric cancer-derived PTX3 promotes macrophage recruitment, which may contribute to gastric cancer-related inflammation.

Chronic inflammation is closely associated with gastric cancer initiation, progression, and metastatic spread (1). For example, persistent infection with *Helicobacter pylori* enables its bacterial toxins to stimulate gastric epithelial cells to produce a wide variety of inflammatory factors including tumor necrosis factor-alpha (TNFα) and interleukins 1, 6 and 8 (IL1, IL6 and IL8), which contribute to tumor-associated inflammation, thus promoting gastric cancer development (2, 3). One key feature of tumor-associated inflammation is the infiltration of leukocytes, and of macrophages in particular (4).

The long form of pentraxin-3 (PTX3) is an inflammatory modulator of innate immunity (5) which is synthesized by inflammatory factors, including lipopolysaccharides, IL1β, and TNFα (6). PTX3 has been implicated in angiogenesis, atherosclerosis, cellular proliferation, and tumor escape from immune surveillance (6-9). Importantly, there exists emerging evidence for elevated PTX3 expression in malignant cancer from multiple tissue types (e.g. glioma, liposarcoma, lung cancer, prostate carcinoma, pancreatic carcinoma, and breast cancer metastatic to bone) that supports a possible role of PTX3 in cancer-related inflammation (10-15).

In the present study, we investigated the direct secretion of PTX3 from gastric cancer cells stimulated by TNFα and the potential significance of PTX3 in macrophage recruitment to gastric cancer, producing an inflammatory microenvironment that promotes the formation of gastric cancer.

**Materials and Methods**

**Cell line and reagents.** HTB135 human gastric cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Pyrrolidine dithiocarbamate (PDTC), SP600125, SB203580, and PD98059 were obtained from Sigma (St. Louis, MO, USA). Recombinant human PTX3, IL1β, IL17, IL23, IL34, and TNFα proteins and antibody to human PTX3 were purchased from R&D Systems (Minneapolis, MN, USA). Antibody to CD11b was obtained from Abcam (Cambridge, MA, USA).

**Immunohistochemistry.** All human patients were treated in accordance to the Declaration of Helsinki. Under protocol no. 2011-0484 approved by the Institutional Review Board of Asan Medical Center (Seoul, Korea), gastric cancer tissue specimens were obtained after surgical removal from 18 patients between 45-73 years of age. Immunohistochemistry (IHC) of frozen sections was performed with...
primary antibody against PTX3 or CD11b using the EnVision™ G|2 Doublestain System, rabbit/mouse (DAB+/Permanent Red) according to the manufacturer's protocol (Dako, Carpinteria, CA, USA). Corresponding rabbit sera were used as negative controls. All slides were counterstained with Mayer’s hematoxylin (Invitrogen, Carlsbad, CA, USA).

Total RNA extraction, cDNA synthesis and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the cells according to the protocol of the RNaseasy Mini Kit (QIAGEN, Hilden, Germany). First-strand cDNA was synthesized using RevertAid First-Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) and amplified using the DreamTaq amplification kit (Thermo Fisher Scientific Inc.). PCR was carried out using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The sequences of primers used included the following: human PTX3, 5’-CAT CTC CTT GCG ATT CTG TTT TG-3’ (sense) and 5’-CCA TTC CGA GTG CTC CTG A-3’ (antisense); human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5’-AGC CAC ATC GCT CAG ACA TAC GAC CAA ATC C-3’ (sense) and 5’-GCC CAA TAC GAC CAA ATC C-3’ (antisense).

Enzyme-linked immunosorbent assay (ELISA). The concentration of the secreted PTX3 protein in conditioned media was determined using a PTX3-specific sandwich ELISA system (R&D Systems) according to the protocols of the manufacturer. Serially diluted

Figure 1. Tumor necrosis factor-alpha (TNFα) elevates long form of pentraxin-3 (PTX3) expression in bone-metastatic human gastric cancer cells. A: Elevated PTX3 expression in advanced human gastric cancer. CD11b and PTX3 expressions were analyzed by immunohistochemical staining (and counterstaining with hematoxylin) in gastric tumor tissues obtained from sequentially staged patients with gastric cancer (stage I-IV). IgG indicates rabbit control antibodies. Scale bar, 200 μm B: HTB135 cells were treated with various cytokines (10 ng/ml TNFα, interleukins 1β, 17, 23, or 34 (IL1β, IL17, IL23, or IL34)) for 24 h. The mRNA expression level of PTX3 was determined by reverse transcription-polymerase chain reaction (RT-PCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. C: The PTX3 protein levels in culture media obtained from HTB135 cells were analyzed using an ELISA assay. D: Following TNFα treatment for 24 h at the indicated concentrations, the PTX3 mRNA expression level was determined by RT-PCR. E: PTX3 protein expression levels following TNFα treatment were determined as described in panel C. Bars indicate the mean and standard deviation (SD) of triplicate samples. *Significantly different at p<0.05 in comparison to untreated cells. F: HTB135 cells were pre-treated with the vehicle (dimethyl sulfoxide), PD98059 (10 μM), SB203580 (10 μM), SP600125 (10 μM), or pyrrolidinedithiocarbamate (PDTC) (10 μM) to determine the role of activation of extracellular signal-regulated protein kinase (ERK), p38 mitogen-activated protein kinase (MAPK), JNK, and NF-κB in TNFα-mediated PTX3 expression for 30 min prior to TNFα stimulation (10 ng/ml TNFα) for 24 h. PTX3 mRNA levels were analyzed using RT PCR. Veh, Vehicle; PD, PD98059; SB, SB203580; SP, SP600125; PT, PDTC.
recombinant human PTX3 was used as the standard. All samples in each analysis were examined in triplicate.

Small siRNA and transfection. The transfection of small interfering RNA (siRNA) targeting PTX3 was performed as previously described (15). A combination of the four selected sequences of siRNA oligonucleotides, SMARTpool siRNA-targeting PTX3 (ONTARGET plus human PTX3), and control siRNA were obtained from GE Dharmacon (Lafayette, CO, USA). siRNAs were transfected into HTB135 cells using RNAiMAX (Invitrogen Life Technologies, Carlsbad, CA, USA). Silencing efficiency was determined using RT-PCR and ELISA as described above.

Cell migration scratch assay. Straight scratches were made on a monolayer of HTB135 cells incubated in 48-well plates using a sterile 200 μl pipette tip followed by treatment with recombinant PTX3 (R&D Systems, Minneapolis, MN, USA) (0, 10, or 100 ng/ml), and the cells were allowed to migrate for 12 hours at 37˚C. Alternatively, HTB135 cells were transfected with control or PTX3 siRNA, allowed to grow to a monolayer and then the wounds were made. siRNA-transfected HTB135 cells were stained with CellTracker Green (CMFDA; Invitrogen Life Technologies) according to the manufacturer’s protocol and photographed using the LSM700 fluorescence microscope system (Carl Zeiss, Jena, Germany). The areas of the remaining scratches were calculated using Adobe Photoshop CS4 software (Adobe Systems, Mountain View, CA, USA). Wound closure was determined as the percentage of the wound area that filled in comparison to the respective controls (regarded as 100%). Each experiment was performed in triplicate.

Macrophage isolation and transwell migration assay. Mouse bone marrow cells were obtained from the femur and tibia of 6-week-old female mice and cell transmigration assays were conducted using 24-well transwell chambers (Costar, Corning, NY, USA) as previously described (15). In brief, HTB135 cells (4×10⁵) were cultured in the lower compartments of a 5 μm-pore transwell system with different concentrations of PTX3 (0, 10, or 100 ng/ml). Macrophages (2×10⁶) labeled with CMFDA (Invitrogen) were overlaid onto the upper chamber. After incubation for 6 hours, the number of macrophages that migrated into the lower chamber was determined using fluorescence microscopy (LSM700; Carl Zeiss, Jena, Germany). For the PTX3-silencing experiment, either PTX3-targeting or control siRNAs were introduced into the HTB135 cells in the lower chamber by transfection followed by TNFα or vehicle treatment.

In vitro binding assay. HTB135 cells were plated onto 96-well plates at 2×10⁴ cells/well in growth medium and the cultures were incubated for one day. Macrophages (1×10⁶ cells) were labeled with fluorescent dyes (CMFDA; Invitrogen) and layered onto HTB135 cells. After extensive washing, the remaining fluorescence was quantified as a measure of macrophage binding to HTB135 cells using a fluorescent plate reader (Spectra GEMINI X; Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis. All quantitative experiments were performed at least in triplicate, and data were expressed as the mean±standard deviation of a single representative experiment. Mean values between groups were compared, and statistical significance was determined using the student’s t-test. A p-value of less than 0.05 was considered statistically significant.

Results

Elevation of PTX3 protein in inflammatory sites of human advanced gastric cancer. We investigated whether elevated PTX3 protein expression is correlated with infiltration of inflammatory cells in human patients. Histological analysis of CD11b-stained sections revealed that the number of tumor-infiltrating myeloid cells (16) was much higher in patients with gastric tumors in stage III than those of other stages (Figure 1A). PTX3 expressed in the stromal cells adjacent to tumors, which was further increased in patients with stage III advanced gastric tumors in comparison to patients with stage I–II or IV (Figure 1A). These findings demonstrate that PTX3 expression is correlated with gastric cancer-related inflammation.
TNFα up-regulates PTX3 expression in human gastric cancer cells. To investigate whether gastric cancer HTB135 cells induce PTX3 expression in response to pro-inflammatory cytokines, HTB135 cells were treated with TNFα, IL1β, IL17, IL23, and IL34. TNFα and IL1β increased the transcription of PTX3 mRNA in HTB135 cells, whereas IL17, IL23 and IL34 did not affect PTX3 mRNA expression (Figure 1B). Of note, ELISA analysis confirmed that the amount of secreted PTX3 protein was significantly elevated in HTB135 cells in response to TNFα (Figure 1D). TNFα increased PTX3 mRNA (Figure 1D) and protein expression (Figure 1E) of HTB135 cells in a dose-dependent manner. This effect of TNFα on PTX3 mRNA expression was effectively reduced by the nuclear factor kappa B (NFκB) inhibitor PDTC (Figure 1F), indicating that activation of NFκB is involved in TNFα-induced PTX3 expression in gastric cancer cells.

PTX3 promotes gastric cancer cell migration. Inflammatory mediators such as TNFα, IL1β, and IL6 increase the invasive capacity of cancer cells (17). Given that TNFα-mediated up-regulation of PTX3, we reasoned that PTX3 could be a mediator facilitating the migratory potential of gastric cancer cells. The administration of recombinant human PTX3 protein promoted migratory activities in HTB135 cells (Figure 2A and B). In contrast, PTX3-silenced HTB135 cells (Figure 2C) demonstrated defective migration in comparison with control siRNA-transfected cells (Figure 2D and E). These results suggest that PTX3 has a promotory effect on gastric cancer cell migration.

PTX3 promotes chemotaxis of macrophages and their subsequent binding to gastric cancer cells. Macrophage recruitment enhanced by inflammatory cytokines and chemokines derived from cancer cells is an important determinant of tumor progression and metastasis (18-20). The prominent increase in PTX3 expression in response to TNFα may suggest a potential role of this protein in macrophage chemotaxis. To test this possibility, we...
performed a transwell assay. HTB135 cells in the lower chamber were treated with different concentrations of PTX3 (0, 10, or 100 ng/ml), and macrophages were loaded into the upper chamber. We found that PTX3 significantly enhanced macrophage chemotaxis in a dose-dependent manner (Figure 3A) and subsequent binding of macrophages to HTB135 cells (Figure 3B).

**PTX3 silencing suppresses macrophage chemotaxis and subsequent binding to gastric cancer cells.** To further confirm the involvement of PTX3 in macrophage migration and binding to gastric cancer cells, **PTX3 silencing was performed.** PTX3 knock-down was validated using an ELISA because 70% of the secreted PTX3 protein was suppressed in PTX3 siRNA-transfected HTB135 cells compared to control siRNA-transfected cells (Figure 4A). **PTX3 silencing in HTB135 cells markedly reduced macrophage migration to gastric cancer cells following TNFα treatment (Figure 4B).** In addition, **PTX3 silencing in HTB135 cells suppressed the binding of macrophages to gastric cancer cells (Figure 4C).** These results demonstrate that PTX3 secreted from HTB135 cells functionally stimulates the chemotactic migration of macrophages and subsequent macrophage binding to gastric cancer cells.

**Discussion**

Various types of cancer cell are able to metastasize to distant parts of the body while producing inflammatory cytokines (*e.g.*, TNFα, IL1β, and IL6) that promote the capability of tumor cells to infiltrate, migrate, and metastasize by potentially upregulating the expression of chemokine receptors, thereby resulting in a vicious and self-reinforcing cycle (17, 21). The fact that PTX3 expression is stimulated by inflammatory cytokines, including TNFα and IL1β, in various tumor types (15, 22, 23) suggests its possible role as an inflammatory mediator involving tumor progression in such inflammatory conditions. We, herein, found that gastric cancer-derived PTX3 increased the migratory potential of gastric cancer cells, consistent with previous results in other type of cancer, including pancreatic carcinoma cells (13) and breast cancer (15). Overall, **PTX3 silencing leads to suppression of the migratory activity of HTB135 gastric cancer cells.** It is possible that PTX3 may induce the expression of chemokine receptors related to metastasis; however, the exact nature of the role of PTX3 in regulation of chemokine receptors remains unclear, and requires further investigation regarding the mechanisms underlying PTX3-mediated metastasis of gastric cancer cells.

Solid tumors communicate reciprocally with cells in their stroma, including with macrophages, to mediate tumor progression and metastasis by the secretion of several mediators such as TNFα, eventually leading to recruitment of macrophages (24, 25). Macrophages that have entered inflamed gastric tissue also predominantly produce TNFα and subsequently recruit additional macrophages, playing a crucial role in tumor progression and metastasis (26, 27). In response to inflammatory stimuli, macrophages also provide various compounds ranging from inflammatory cytokines, mutagenic oxygen, and angiogenic factors that facilitate angiogenesis, matrix breakdown, and tumor cell motility, all of which are elements of tumor progression and metastasis (28). Accordingly, we observed that TNFα-mediated PTX3 expression enhanced the potential of macrophages to migrate to gastric cancer cells, and **PTX3 silencing abrogated the motility capacity of macrophages similar to its role in metastatic breast cancer cells that we previously reported (15).** These results suggest that PTX3 derived from metastatic tumor cells serves as a tumor-promoting factor by facilitating cancer cell migration and recruitment of macrophages, which are responsible for tumor progression and metastasis. In contrast, Deban *et al.* reported that PTX3 binds P-selectin and attenuates neutrophil recruitment at sites of inflammation (29). Although we have shown that PTX3 promotes chemotactic migration and subsequent binding of macrophages to gastric cancer cells, the present study has certain limitations, as we have not elucidated the precise mechanism of macrophage chemotaxis elicited by PTX3. To clarify these contradictory findings and the underlying mechanism of PTX3-mediated chemotaxis of macrophages, further studies are required. It is well-established that tumor-associated macrophages acquire M2-like properties when in the stroma of many tumors, and the accumulation of these cells is frequently associated with the progression of several types of cancers (30), whereas infiltration by M1 macrophages elicited protective immune responses against the tumor (4). Further studies are needed to determine if PTX3 produced from gastric cancer cells is responsible for the chemotaxis of M2 macrophages.

In conclusion, we have herein demonstrated that in metastatic gastric cancer cells, TNFα stimulates PTX3 expression via the NFκB pathway, and PTX3 acts as an inducer of gastric cancer cell migration and chemotactic migration of macrophages. These findings provide a better understanding over the mechanisms by which PTX3 exerts its effects on the progression of gastric cancer-related inflammation. Our work raises the possibility for clinical interventions targeted at the inflammation associated with gastric cancer.

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Conflicts of Interest

The Authors report no conflict of interest in regard to this study. The Authors alone are responsible for the content and writing of the article.

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