Interaction of coagulation factors and tumor-associated macrophages mediates migration and invasion of gastric cancer

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Abundant macrophage infiltration and increased expression of coagulation factors have been observed in cancer patients. The aim of the present study was to determine how the interaction between activated coagulation factors and monocytes/macrophages contributes to gastric cancer (GC) cell migration and invasion. We assessed cytokine/chemokine production of coagulation-factor-treated macrophages by ELISA. The effects of the interaction between coagulation factors and tumor-associated macrophages (TAM) on GC cell migration and invasion were determined by in vitro migration and invasion assay. In addition, we used an in vitro co-culture system of GC cells/TAM treated by coagulation factors to estimate the effect of coagulation factor/TAM interaction on the human umbilical vein endothelial cell line (HUVEC). We found that the M2-like phenotype of interleukin (IL)-4(high), IL-10(high), transforming growth factor (TGF)-α(high), tumor necrosis factor (TNF)-α(high) was exhibited when the human monocytic cell line THP-1 was stimulated by coagulation factors III (TF), VIIa (FVIIa) and XIIa (FXIIa). For the migration assay, the GC cells (BGC-823 or SGC-7901) that were co-cultured with activated coagulation factor/TAM both showed increased migration. For the invasion assay, both BGC-823 and SGC-7901 cells co-cultured with TF/TAM showed increased invasion. We also found that TAM activated by coagulation factors could induce vascular endothelial growth factor/MMP-9 expression, which could promote invasion of GC cells. The HUVEC co-cultured with TAM (PMA-treated THP-1 macrophages co-cultured with GC cells) expressed high levels of FXIIa. In conclusion, coagulation factors might facilitate GC cell migration and invasion by transforming macrophages toward TAM-like cells. Interaction of coagulation factors and TAM mediates migration and invasion of GC. (Cancer Sci 2011; 102: 336–342)

Despite its decreasing incidence, gastric cancer (GC) is still the second leading cause of cancer-related death worldwide, particularly in Asian countries.¹,² Peritoneal metastasis is the most frequent event in recurrent GC and occurs in 34% of patients with recurrence, even after curative resection of the primary tumor.³,⁴ Therefore, new approaches to the treatment of metastatic GC have become a hot topic. In addition to clonal selection and the predetermined metastatic potential of cancer cells, there is increasing evidence that the microenvironment modifies cancer cell metastasis.¹,³–⁶ Thus, one approach to the treatment of metastatic GC is to identify novel targets based on cancer cell–tumor microenvironment interactions.⁷–¹¹ Tumor stroma consists of activated fibroblasts (myofibroblasts) and smooth muscle, endothelial and inflammatory cells, including macrophages. Macrophages that migrate to tumor stroma are called tumor-associated macrophages (TAM). The role of TAM in tumor progression is complicated. Although activated macrophages might have antimetastasis ability, tumor cells are reported to escape the antimetastasis activity of TAM.¹² It has also been reported that TAM are not only abundant in epithelial cancers, but also involved in cancer progression.¹³–¹⁶ Indeed, removal of macrophages by genetic mutation has been shown to reduce tumor progression and metastasis.¹³ Tumor-associated macrophages of the M2 phenotype secrete several growth factors (e.g. transforming growth factor [TGF]-β and vascular endothelial growth factor [VEGF]) that can promote tumor growth.¹⁷–²¹ Experimental data have indicated that ablation of macrophage function or inhibition of macrophage infiltration into experimental tumors inhibits tumor growth and metastasis.²² Therefore, it has become clear that TAMs are active players in the process of tumor progression and invasion.

The history of a known association between blood coagulation and cancer dates back to 1865 when Armand Trousseau observed that patients who presented with idiopathic venous thromboembolism frequently had an underlying occult cancer and vice versa.²³ The association has only recently become more apparent. Previously, researchers have focused on how tumors activate blood coagulation and how to overcome it. However, the underlying mechanism by which coagulation factors promote tumor cell growth, invasion, metastasis and angiogenesis has become a hot topic in the field of cancer research.

A remarkable feature of advanced GC is the presence of widespread metastases at the time of the initial diagnosis. Unfortunately, the mechanisms that underlie seeding, spreading and progression remain elusive. In the present study, we co-cultured M2-polarized human THP-1 macrophages with GC cells and human umbilical vein endothelial cells (HUVEC) without direct contact in a Transwell apparatus. We analyzed the characteristics of M2 macrophages. The interaction of coagulation factors and TAM in mediating GC migration and invasion was also investigated.

Materials and Methods

Cell preparations. Human monocytic cell line THP-1 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin.

Gastric adenocarcinoma cell lines SGC-7901 and BGC-823 and human umbilical vein endothelial cell line HUVEC were kindly provided by the Digestive Surgery Institution, Ruijin Hospital of Shanghai (Shanghai, China). They were cultured in RPMI 1640 supplemented with 10% FBS and maintained at 37°C with 5% CO₂.

Flow cytometry. THP-1 cells were treated with 320 nM PMA for 24 h to induce cells to macrophages and then washed three times with PBS to remove PMA. After blocking human Fc

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receptors (FcRs), the cells were washed and resuspended in PBS supplemented with 1% FBS and 0.01% NaN3. For CD68 staining, the cells were fixed and permeabilized with a BD Cytofix/CytopermTM Fixation/Permeabilization Solution kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells were then incubated with the FITC-CD68 mAb (eBioscience, San Diego, CA, USA). For surface markers, the cells were incubated with FITC-CD206 mAb (BD Biosciences) or PE-CD204 mAb (R&D, Minneapolis, MN, USA). Following the final washing step, labeled cells were analyzed by Guava EasyCyte System (Millipore, Billerica, MA, USA).

**Sandwich ELISA.** Macrophages were treated with serum-free medium supplemented with coagulation factors for another 48 h. The concentration of the coagulation factors was 100 ng/mL coagulation factor III (tissue factor [TF]; Enzyme Research Labs, South Bend, IN, USA), 0.01 nmol/mL coagulation factor VIIa (FVIIa; Enzyme Research Labs) and 0.05 nmol/mL coagulation factor XIIa (FXIIa; Enzyme Research Labs). The culture supernatant was collected at different time intervals. The concentration of tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-10, TGF-β in the supernatants was determined using an ELISA kit from Cusabio Biotech (Carlosbad, CA, USA) according to the manufacturer’s instructions.

**Effect of macrophages on GC cells.** One million THP-1 cells were seeded into the upper insert of a six-well Transwell and treated with 320 nM PMA for 24 h. PMA-containing medium was removed and macrophage cells were washed three times in PBS to remove all PMA. Tissue factor (100 ng/mL), FVIIa (0.01 nmol/mL) or FXIIa (0.05 nmol/mL) were added to the upper insert for another 24 h, respectively. The coagulation-factor-treated macrophages were then co-cultured with GC cells (BGC-823 or SGC-7901) for 6 h. After co-culture, the macrophages were discarded and the GC cells were assayed for migration and invasion. The GC cells co-cultured with untreated macrophages were used as negative controls.

Cell migration was assessed using a 24-well format that contained a Transwell insert (8-μm pore size polycarbonate filter; Corning Life Sciences, Lowell, MA, USA). BGC-823 or SGC-7901 GC cells (2.5 × 10⁴) were suspended in serum-free RPMI 1640 medium and seeded in the upper chamber, and NIH 3T3-fibroblast-conditioned medium was added to the lower chamber. For each treatment, three wells were used. After incubation with 5% CO₂ for 24 h at 37°C, the non-migrated cells in the upper chamber were removed by wiping with a cotton swab, and the attached migrated cells were fixed and stained with hematoxylin–eosin (HE). Three different fields of cells were photographed with an Olympus microscope and the migrated cells were counted at ×400 magnification in 10 different fields.

The invasion assay was conducted using Transwell cell culture chambers (Millipore). Each insert contained an 8-μm pore size polycarbonate membrane coated with a thin layer of ECM Matrix. BGC-823 or SGC-7901 GC cells (1 × 10⁵) were seeded onto Matrigel with serum-free RPMI 1640. NIH 3T3-fibroblast-conditioned medium was added to the lower chamber. After 48 h of incubation at 37°C in 5% CO₂, cells on the upper surface of the inner chamber were removed with cotton swabs. Invading cells that adhered to the lower surface of the membrane were fixed and stained with HE. The invading cells were counted at ×400 magnification in 10 different fields for each insert. The experiments were repeated three times.

To analyze the possible molecular mechanism involved in GC cell invasion, the co-cultured GC cells were collected and subjected to western blot assay for VEGF and MMP-9. We also used specific inhibitors to inhibit the activity of these molecules (chem, San Diego, CA, USA), respectively, then subjected to *in vitro* invasion assay.

**Interaction between macrophages and HUVEC.** To measure the direct effect of TAM on HUVEC, *in vitro* co-culture system was used.²³ Briefly, 1 × 10⁴ BGC-823 or SGC-7901 cells were seeded in the lower chamber, 1 × 10⁵ macrophages treated by TF, FVIIa or FXIIa were grown in the upper chamber, and the bottom of the Transwell consisted of a membrane (0.4-μm pore size) that was permeable to liquids but not to cells. After incubation at 37°C in 5% CO₂ for 48 h, the TAM in the upper chamber were collected and seeded in another lower chamber of the Transwell, and at confluence the medium was changed to serum-free RPMI 1640. Meanwhile, HUVEC were grown in the upper chamber, and at confluence the medium was changed to serum-free RPMI 1640. Cells in this co-culture system were allowed to grow for the indicated time. Culture supernatants were collected at different time intervals to detect the concentration of the coagulation factor expressed by HUVEC with an ELISA kit (Cusabio Biotech). For each treatment, three wells were used.

The HUVEC were also collected and total RNA was isolated with Trizol (Invitrogen) and reverse-transcribed using the SuperScript II RNase-Reverse Transcriptase System (Invitrogen). The cDNA was then subjected to PCR with specific primers for TF, FVII and FXII. The sequence for TF was: forward, 5'-GA-ACCCAAAAACGTCGATA-3'; and reverse, 5'-GAA GAC CCG TGC CAA GTA-3'. The sequence for FVII was: forward, 5'-GCC CCA CCA ACC ACC ACA CAA-3'; and reverse, 5'-GCC GCT GAC CAA TGA GAA-3'. The sequence for FXII was: forward, 5'-TGA CCA CAA CAA GCC GGC-3'; and reverse, 5'-AAA GAT GAG TCC CTC AGC AGA AA-3'. The sequence of the primers used for β-actin was: forward, 5'-CTA CAA TGA GCT GCG TGT GCC-3'; and reverse, 5'-CAG GTC CAG ACG CAG GAT GCC-3' (Invitrogen).

**Statistical analysis.** Statistical analyses of the data were performed with SPSS version 13.0 software (SPSS; Chicago, IL, USA). If the results were distributed normally, the two independent sample t-test was used for comparison. For comparisons between groups of more than two unpaired values, we used one-way ANOVA. If an ANOVA F value was significant, post-hoc comparisons were performed between groups. If results were not normally distributed, the Mann–Whitney U-test was used to compare two groups of unpaired values, whereas for comparisons between groups of more than two unpaired values, we used the Kruskal–Wallis test. All P values resulted from the use of two-sided statistical tests and P < 0.05 was considered significant.

**Results**

**PMA treatment differentiated THP-1 cells to M2 macrophages.** Human THP-1 cells are widely used as models for monocyte/macrophage differentiation.²³ When treated with PMA for 24 h, the THP-1 cells (Fig. 1A) quickly stopped proliferating, attached and differentiated to monocytes, and then subsequently to macrophages (Fig. 1B). The macrophages had a high degree of functional plasticity because they could change their functional profiles (from M1 to M2 or M2 to M1) repeatedly, depending on the cytokines (Th1 or Th2) to which they were exposed.²⁴ The PMA-treated THP-1 macrophages (CD68 positive, Fig. 2A) exhibited significant expression of M2 macrophage surface markers CD206 (mannose receptor, Fig. 2B; Fig. 2C was the isotype control of FITC). The PMA-treated THP-1 macrophages also exhibited significant expression of CD204 (scavenger receptor A, Fig. 2D); Fig. 2E was the isotype control of phycoerythrin (PE).

**Effect of coagulation factors on TAM.** M2-polarized THP-1 macrophages were activated by TF, FVIIa or FXIIa. The cytokines associated with tumor metastasis and inductions of

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immune tolerance secreted by macrophages were detected by ELISA. The inductions IL-4 (Fig. 3A), IL-10 (Fig. 3B), TGF-β (Fig. 3C) and TNF-α (Fig. 3D) of the PMA-treated THP-1 macrophages induced by TF, FVIIa or FXIIa showed in different profiles. When compared to negative controls, macrophages activated by TF had significantly high levels of IL-10, but low levels of IL-4, TGF-β and TNF-α; macrophages activated by FVIIa had significantly high levels of IL-10 and TNF-α, but low levels of IL-4 and TGF-β; macrophages activated by FXIIa had significantly high levels of IL-4, TGF-β and TNF-α, but had no difference in IL-10.

TAM activated by coagulation factors induce migration and invasion of GC cells. The PMA-treated and M2-polarized THP-1 macrophages treated with TF, FVIIa or FXIIa were co-cultured with GC cells. For the in vitro migration assay, BGC-823 or SGC-7901 cells that had been co-cultured with TAM treated with TF, FVIIa or FXIIa for 24 h showed an increased number of migrated cells (compared with the negative controls, P < 0.05; Fig. 4). For the in vitro invasion assay, BGC-823 or SGC-7901 cells were co-cultured with TAM activated by coagulation factors for 48 h. The BGC-823 and SGC-7901 cells co-cultured with TAM induced by TF showed an increased number of invading cells (compared with negative controls, P < 0.05; Fig. 5A,B). Micrographs show cells invading through 8-μm pores on the lower side of the filters in correspondence to the above groups (Fig. 5C,D). Gastric cancer cells co-cultured with TAM induced by FVIIa or FXIIa showed no difference in invasion when compared with negative controls.

TAM activated by coagulation factors induce VEGF/MMP-9 expression. To detect the molecular mechanisms involved in GC cell migration and invasion, we determined VEGF/MMP-9 expression and examined the effects of the specific inhibitors against these molecules. We found that macrophages significantly induce the expression of VEGF and MMP-9 of GC cells (Fig. 6A). When VEGF/MMP-9 activity was blocked by specific inhibitors, the number of invaded cells was obviously decreased (Fig. 6B,C). Taken together, macrophages activated by coagulation factors might induce VEGF/MMP-9 expression in GC cells and subsequently promote the invasiveness of GC cells.
TAM increase coagulation factor expression of HUVEC. We used an in vitro co-culture system of BGC-823 or SGC-7901 cells and TAM activated by TF, FVIIa or FXIIa. The HUVEC co-cultured with TAM (GC cells co-cultured with PMA-treated THP-1 macrophages) expressed high mRNA levels of TF, FVIIa and FXIIa (Fig. 7A). Also, HUVEC co-cultured with TAM expressed high levels of FXIIa in supernatants, but there were no significant differences in TF and FVIIa between TAM-induced HUVEC and negative controls (Fig. 7B–D).

Discussion

It has been reported that patients with idiopathic venous thromboembolism frequently have underlying occult cancer and vice versa.\(^{25}\) Previously, researchers have focused on how tumors activate blood coagulation and how to overcome it. However, some coagulation factors have been reported to have a role in tumor progression,\(^{25}\) but the underlying mechanism by which coagulation factors promote tumor cell growth, invasion, metastasis and angiogenesis is still unknown, especially in GC.

Macrophages are the key cells that are involved in chronic inflammation and they can be phenotypically polarized by the microenvironment to mount specific functional programs.\(^{26}\) Macrophages in the tumor microenvironment are defined as TAM and exhibit M2 characteristics.\(^{27}\) There is considerable controversy as to whether TAM promote or inhibit tumor progression. Originally it was thought that the main function of TAM was to exert direct cytotoxic effects on tumor cells, phagocytose apoptotic/necrotic cell debris and present tumor-associated antigens to T cells. However, recent studies suggest that an important characteristic of macrophages is their potential for angiogenic activity and promotion of tumor growth and metastasis.\(^{28}\) Activated macrophages produce various factors that induce angiogenesis in wound repair\(^{29}\) and in chronic inflammatory diseases.\(^{30}\) Tumor-associated macrophages demonstrate several pro-tumor functions, including secretion of growth factors and matrix proteases, promotion of angiogenesis and suppression of adaptive immunity.\(^{12,18}\) It has been reported that TAM infiltration into tumor tissue correlates significantly with tumor vascularity in human esophageal and gastric cancers.\(^{31,32}\) Ishigami et al.\(^{33}\) have also found direct associations between the degree of TAM infiltration and depth of tumor invasion, nodal status and clinical stage in GC. The underlying mechanism of promotion of cancer metastasis by TAM has become a hot topic in cancer research. Macrophage recruitment is mediated by a variety of chemoattractants, including monocyte chemoattractant protein-1 and macrophage inflammatory protein 1α. However, the role of TAM activated by coagulation...
factors in GC cell metastasis and invasion is still unclear. In the present study, we found that a M2-like phenotype of IL-4 high, IL-10 high, TGF-β high and TNF-α high was exhibited when THP-1 were stimulated by TF, FVIIa or FXIIa. IL-4, IL-10, TNF-α and TGF-β were reported to promote cancer growth and invasion. Therefore, we think that coagulation factors might induce TAM to release various factors to support tumor cell growth and invasion.

Gene chip analysis has revealed an increased level of FXII mRNA in the peritoneum of epithelial ovarian cancer, which suggests that FXII plays a role in metastasis and ascites formation in the peritoneal cavity. To confirm the mechanism by which coagulation factors promote GC cell growth and invasion, we co-cultured GC cells with TAM that had been activated by coagulation factors and then analyzed the migration and invasion of GC cells. For the in vitro migration assay, BGC-823 or SGC-7901 GC cells co-cultured with TAM activated by TF, FVIIa or FXIIa showed increased migration. For the in vitro invasion assay, we found that BGC-823 and SGC-7901 cells co-cultured with TAM activated by TF showed increased invasion. However, GC cells co-cultured with TAM activated by FVIIa or FXIIa showed no difference when compared with negative controls. As a result, we suggest that coagulation factors can activate TAM to promote GC cell growth, migration and invasion, and different coagulation factors could have different effects on GC cells. We found that TF, FVIIa and FXIIa can activate TAM to promote GC cell migration, and TF can activate TAM to promote GC cell invasion. Upon activation by cancer cells, TAM can release diverse growth factors, proteolytic enzymes and cytokines. In clinical studies, high numbers of TAM have been shown to correlate with high vessel density and tumor progression. It has been reported that FXII might facilitate

Fig. 4. Migration potential of gastric cancer (GC) cells was detected by Transwell migration assay. (A) Number of migrating BGC-823 cells. (B) Number of migrating SGC-7901 cells. Negative, GC cells co-cultured with macrophages not activated by coagulation factors; tissue factor (TF), GC cells co-cultured with tumor-associated macrophages (TAM) activated by TF; FVIIa, GC cells co-cultured with TAM activated by FVIIa; FXIIa, GC cells co-cultured with TAM activated by FXIIa. Compared with negative controls, *P < 0.05, **P < 0.01 and ***P < 0.001.

Fig. 5. Invasion potential of gastric cancer (GC) cells was detected by Matrigel invasion assay. (A,B) Data are expressed as the mean number of invading cells per field (average of 10 fields per filter). Negative, GC cells co-cultured with macrophages not activated by coagulation factors; tissue factor (TF), GC cells co-cultured with tumor-associated macrophages activated by TF. Compared with negative controls, **P < 0.01. (C,D) Micrographs show cells invading through 8-μm pores on the lower side of the filters corresponding to the above groups.
Fig. 6. Tumor-associated macrophages (TAM) activated by coagulation factors induce vascular endothelial growth factor (VEGF)/MMP-9 expression. (A) The TAM activated by coagulation factors induced secretion of VEGF and MMP-9 from gastric cancer (GC) cells significantly. Control, GC cells co-cultured with TAM not treated by coagulation factor; TF, GC cells co-cultured with TAM treated by coagulation factor TF. (B,C) VEGF and MMP-9 specific inhibitor significantly decrease macrophage-induced invasiveness of GC cells (**P < 0.01). Control, GC cells not treated by specific inhibitor after co-cultured with coagulation-factor-activated TAM; VEGF inhibitor, GC cells treated by VEGF inhibitor after co-culturing with coagulation-factor-activated TAM; MMP-9 inhibitor, GC cells treated by MMP-9 inhibitor after co-culturing with coagulation-factor-activated TAM.

Fig. 7. Effect of coagulation-factor-activated tumor-associated macrophages (TAM) on human umbilical vein endothelial cells (HUVEC). We used an in vitro co-culture system of BGC-823 or SGC-7901 cells and TAM activated by tissue factor (TF), FVIIa or FXIIa. The TAM were co-cultured with HUVEC for another 48 h. We detected the levels of coagulation factors in HUVEC cells and the collected supernatant. (A) mRNA levels of coagulation factors in HUVEC cells. Negative, TAM not co-cultured with GC cells; BGC-823, TAM co-cultured with BGC-823; SGC-7901, TAM co-cultured with SGC-7901. (B–D) Concentration of TF, FVIIa and FXIIa in the collected supernatant.
epithelial ovarian cancer (EOC) cell metastasis by transforming monocytes/macrophages toward TAM-like cells. Also, conditioned medium of FXII-stimulated monocytes/macrophages significantly increases EOC cell invasion. We also found that macrophages activated by coagulation factors might induce VEGF/MMP-9 in GC cells and subsequently increase the invasiveness of GC cells. Therefore, we suggest that TAM activated by coagulation factors might induce VEGF/MMP-9 expression, which could promote migration and invasion of GC cells.

To confirm the interaction between angiogenesis and coagulation factors, we assessed the direct effect of macrophages stimulated by GC cells on HUVEC. We used an in vitro co-culture system of BGC-823 or SGC-7901 cells and TAM treated by TF, FVIIa or FXIIa. We found that HUVEC co-cultured with TAM excreted high levels of FXIIa. However, there were no significant differences in TF and FXIIa between TAM-induced HUVEC and negative controls. Therefore, we suggest that coagulation factors can activate TAM to promote GC cell invasion by increasing angiogenesis, and conversely, GC cells can activate TAM to promote HUVEC secretion of coagulation factors. Therefore, the interaction of coagulation factors and TAM mediates metastasis of GC.

In summary, we found that the M2-like phenotype of IL-4(high), IL-10(high), TGF-β(high) and TNF-α(high) was exhibited when macrophages were stimulated by TF, FVIIa or FXIIa. This suggests that coagulation factors act as novel mediators that stimulate macrophages towards the M2 phenotype and promote GC cell growth and invasion. This newly found relationship between coagulation factors and macrophages could help to understand the role of the coagulation cascade and inflammatory cells in the tumor microenvironment, and facilitate further development of new therapeutic strategies for GC that target coagulation factors and macrophages. Gastric cancer cells can activate TAM to promote HUVEC secretion of coagulation factors and this interaction mediates migration and invasion of GC. However, the role of the specific coagulation factors and their mechanism of action require elucidation in further studies.

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Disclosure Statement

The authors have no conflict of interest.

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