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High-mobility group box-1 contributes tumor angiogenesis under interleukin-8 mediation during gastric cancer progression

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Many soluble factors are involved in tumor angiogenesis. Thus, it is valuable to identify novel soluble factors for effective control of tumor angiogenesis in gastric cancer (GC). We investigated the role of extracellular high-mobility group box-1 (HMGB1) and its associated soluble factors in the tumor angiogenesis of GC. Clinically, we measured serum levels of HMGB1 and GC-associated cytokines/chemokines using GC serum samples (n = 120), and calculated microvessel density (MVD) by CD34 immunostaining using human GC tissues (n = 27). Then we analyzed the correlation of serum HMGB1 levels with MVD or that with cytokine/chemokine levels by linear regression. As *in vitro* angiogenesis assay for HMGB1, HUVEC migration and capillary tube formation assay were carried out using different histological types of human GC cells (N87 and KATOIII). CD34-positive microvessels were detected from early GC, but MVD increased according to GC stages, and were closely correlated with serum HMGB1 levels (R = 0.608, P = 0.01). The HUVECs cultured in conditioned media derived from rhHMGB1-treated or HMGB1-TF GC cells showed remarkably enhanced migration and tube formation activities. These effects were abrogated by anti-HMGB1 antibody or HMGB1 siRNA in both N87 and KATOIII cells (all P < 0.05). Among tested cytokines/chemokines, interleukin-8 (IL-8) was the most remarkable cytokine correlated with serum HMGB1 (P < 0.001), and enhanced HUVEC migration and tube formation activities by rhHMGB1 or HMGB1-TF were significantly reversed by IL-8 inhibition. These results indicate overexpressed HMGB1 contributes to tumor angiogenesis through IL-8 mediation, and combined targeting of HMGB1 and IL-8 can control tumor angiogenesis in GC.

Gastric cancer remains the second leading cause of cancer-related mortality worldwide, even though its incidence has decreased over the past century.[1,2] The overall 5-year survival rate of GC is approximately 20% because AGC is associated with high rates of recurrence and metastasis,[2] even after radical resection and aggressive perioperative or adjuvant treatment.[3]

Metastasis is a multistage process involving cancer emigration/invasion, intravasation, circulation, extravasation, and establishment at new sites. Tumor angiogenesis plays a critical role during this multistage process of metastasis in many solid cancers;[4,5] tumor angiogenesis facilitates cancer cells to easily access into blood circulation by providing adequate blood vessel supply. In the complex regulation of angiogenesis, many pro-angiogenic factors are involved.[6–8] Therefore, tumor angiogenesis and its associated soluble factors are highly relevant targets for controlling the metastasis of many solid tumors. Recent studies have shown that tumor-derived angiogenic factors are closely associated with hematogenous metastasis and poor prognosis of many cancers,[6–8] and pharmacologic blockade of angiogenesis by inhibiting these soluble pro-angiogenic factors is a promising strategy for decreasing cancer recurrence and metastasis.[9,10]

A group of molecules that may act as mediators of angiogenesis are the so-called high-mobility group proteins. High-mobility group box-1, known as a pro-inflammatory cytokine, is an important member of the high-mobility group superfamily. It plays an important role in angiogenesis in various situations.[11–14] Recently, many studies have reported that overexpressed HMGB1 is involved in tumor development and metastasis through its ability to mediate tumor-associated inflammation, to promote cell migration and to promote angiogenesis.[15–18] High-mobility group box-1 is overexpressed in approximately 85% of GCs, and high levels of serum HMGB1 are associated with advanced stage and poor prognosis of GC.[19,20] Because HMGB1 is also a potent soluble angiogenic factor in solid tumors,[17,18] extracellular HMGB1 overexpression may contribute to tumor angiogenesis in GC. However, the role of HMGB1 in tumor angiogenesis in GC remains unclear.

In the present study, we investigated the role of extracellular HMGB1 in tumor angiogenesis and determined its associated soluble factors in GC.

Materials and Methods

Cell lines and culture. In the current study, we used NCI-N87 cells as a gastric adenocarcinoma cell line and KATOIII cells as a gastric signet-ring cell carcinoma cell line. These two different types of human GC cells (Korean Cell Line Bank, Seoul, Korea) were cultured in RPMI-1640 (Gibco-Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS) (Gibco-Invitrogen) and 1% antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin; Gibco-Invitrogen) in mycoplasma-free systems under a humidified atmosphere with 5% CO_2 at 37°C.

Primary HUVECs were purchased from Lonza (Basel, Switzerland) and cultured in endothelial cell growth medium (Lonza) under equivalent conditions to those of GC cells. All cells used in experiments underwent fewer than eight passages after resuscitation. The identity and uniqueness of cell lines were confirmed by DNA fingerprinting analyses with short tandem repeat markers.

Clinical sample collection. Serum samples were obtained from a total of 120 GC patients; 40 samples from patients with EGC, 40 samples from patients with AGC-M0 and 40 samples from patients with AGC-M1 at Yonsei University Health System (Seoul, Korea). All disease entities were histologically confirmed. Among 120 GC patients, 27 patients (10 EGC, 10 AGC-M0, and 7 AGC-M1) were selected to obtain tissue samples. Gastric cancer stage was analyzed according to the American Joint Committee on Cancer's TNM staging classification for GC. Clinical sampling was approved by the Institutional Review Board of the Yonsei University Health System for human projects according to the principles of the Helsinki Declaration (No. 4-2011-0609), and written informed consent was obtained. Patients with other chronic diseases or cancers were excluded. Blood samples were collected before any treatment and stored at −80°C as serum fractions until analysis.

Reagents. Human recombinant HMGB1 protein was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-HMGB1 chicken IgY neutralizing polyclonal antibody (anti-HMGB1 Ab) was purchased from Shino-Test (Tokyo, Japan) and anti-chicken IgY for control (control Ab) was purchased from Abcam (Cambridge, UK).

Plasmids and siRNA. Gastric cancer cell lines were transfected with 1 μg pCMV-SPORT6-HMGB1 plasmid (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea) or 1 μg pCMV-SPORT6 vector (Mock; Invitrogen) in a 24-well culture plate using Lipofectamine LTX and Plus Reagent (Invitrogen). Gastric cancer cell lines were transfected with 0.5 μg control siRNA or siRNA targeting HMGB1 or IL-8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a 24-well culture plate using TransPass R2 Transfection Reagent (New England Biolabs, Ipswich, MA, USA).

Enzyme-linked immunosorbent assay and chemiluminescent immunoassay. Serum HMGB1 levels were measured using the commercially available HMGB1 ELISA Kit II (Shino-Test Corporation, Kanagawa, Japan) according to the manufacturer's instructions. For evaluating the soluble factors associated with HMGB1-induced angiogenesis in GC, serum levels of 20 different cytokines or chemokines, which are known to be involved in GC progression, were measured by chemiluminescent immunoassay using the commercially available MILLIPLEX MAP Human Cytokine/Chemokine Kit (HCYTMAG60PMX29BK; Millipore, Billerica, MA, USA). Tested cytokines or chemokines included IL-1α, IL-1β, IL-1 receptor antagonist, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, interferon-γ-induced protein 10, monocyte chemotactic protein-1, VEGF, tumor necrosis factor-α, Eotaxin-1, macrophage inflammatory protein-1α and -1β, and epidermal growth factor.

Evaluation of MVD by CD34 immunohistochemical staining. To evaluate the contribution of HMGB1 to tumor angiogenesis in GC tissues clinically, MVD was calculated in tumor sections by quantification of CD34-positive vessels. Briefly, rehydrated paraffin blocks of GC tissue were retrieved using microwaves, and incubated with primary antibody against CD34 (mouse 1:100; Santa Cruz Biotechnology) at 4°C overnight. Immunohistochemical staining was undertaken using the Envision+ system (Dako, Glostrup, Denmark) and counterstained with hematoxylin solution (Merck, Kenilworth, NJ, USA).

The microvessels stained by CD34 were assessed by light microscopy in areas of the tumor section containing highest vascular density (vascular hot spot). The highly vascular areas were identified by scanning tumor sections at low power field (×40 and then ×100 magnification). After five areas of highest neovascularization were identified, CD34-positive vessels was manually counted at ×200 magnification.[21]

Microvessel density was determined as the average counts of the five fields. Each slide was analyzed by two independent investigators, who were blinded to knowledge of HMGB1 expression, histopathological characteristics of tumors, and characteristics of GC patients.

Preparation of CM for evaluation of tumor angiogenesis *in vitro*. For preparation of CM, GC cells (N87 or KATO-III) were cultured in RPMI-1640 supplemented with 1% FBS alone until 30% cell confluence, then cultured in RPMI-1640 supplemented with 1% FBS and rhHMGB1 (10 g/mL), 1% FBS and rhHMGB1/control Ab, or 1% FBS and rhHMGB1/anti-HMGB1 Ab (60 μg/mL) for 72 h. The culture media were collected, centrifuged at 350 *g*, and the supernatants were obtained. Samples were frozen at −20°C until used. Samples from three independent cell cultures were pooled. Control media were collected from supernatants of GC cells cultured in RPMI-1640 supplemented with 1% FBS alone for 72 h without any treatment. Protein contents in CM were evaluated using bicinchoninic acid assay (BCA protein assay kit; Thermo Fisher Scientific, Waltham, MA, USA).

Similarly, mock-transfected GC cells, HMGB1-TF GC cells, HMGB1-TF with control-siRNA-treated GC cells, and HMGB1-TF with HMGB1-siRNA-treated GC cells were cultured in RPMI-1640 supplemented with 1% FBS for 72 h. The culture media were collected and centrifuged at 350 *g*, and then the supernatants were obtained. Samples were frozen at −20°C until used. Samples from three independent cell cultures were pooled.

Endothelial cell migration and capillary tube formation assays. The endothelial cell migration assay and the capillary tube formation assay are widely used common methods for studying angiogenesis *in vitro*.[22] Thus, we carried out HUVEC migration assays and capillary tube formation assays to evaluate HMGB1-induced angiogenesis *in vitro*.

The HUVEC migration assay was undertaken using the BD BioCoat Angiogenesis System: Endothelial Cell Migration kit (BD Biosciences). For pretreatment, HUVECs were cultured in EBM-2 Basal Medium (Lonza) contained no growth factors or supplements for 24 h to exclude the effect of growth factor in EBM-2. After pretreatment, HUVECs were seeded (5×10^4 cells) on the upper chamber of the insert and incubated in 50 mL CM, which was contained in each of the bottom wells as a chemoattractant to HUVECs. After incubation (24 h), cells on the lower side of the insert were fixed, stained with

2 μM calcein AM for 30 min, and counted. The migration indices were calculated as the mean number of cells in three random fields using DP controller software with an IX71 microscope (×100 magnification; Olympus Corporation, Tokyo, Japan).

In vitro capillary tube formation assay was carried out using a Cultrex In Vitro Angiogenesis Assay Tube Formation Kit (Travigen, Gaithersburg, MD, USA). Growth factor-reduced basal membrane extract was added to each well of a 96-well plate and allowed to be polymerized for 1 h at 37°C. Pretreated HUVECs were seeded (2×10^4 cells) in each well and incubated in 50 mL CM for 24 h. After incubation, cells were stained with 2 μM calcein AM for 30 min and capillary tube formation was quantified by measuring the number of complete capillary-like hollow lumina using the DP controller software with an IX71 microscope (×40 magnification; Olympus Corporation). At least three fields per well were examined, and each experimental condition was tested in triplicate.

Statistical analysis. Serum levels of HMGB1 and cytokines/chemokines were expressed as mean values. To compare averages among three groups, one-way ANOVA with the Bonferroni multiple comparison was used. To compare values between two groups, Student's *t*-test (two-tailed) was used. Linear regression analysis was used to evaluate the correlation between serum levels of HMGB1 and MVD, or the correlations between serum levels of HMGB1 and cytokines/chemokines.

All statistical analyses were carried out with IBM SPSS Statistics 23.0 and *P*-values < 0.05 were considered statistically significant.

Results

Clinical evidence of HMGB1 involvement in tumor angiogenesis of GC. To evaluate the involvement of extracellular HMGB1 in tumor angiogenesis of GC clinically, we used immunohistochemical staining for CD34 in 27 human GC tissue samples and calculated the number of CD34-positive microvessels in the tumor area. We then compared the CD34-positive MVD according to GC stage (Fig. 1). We also analyzed the correlation between serum HMGB1 levels and MVD by performing linear regression analysis (Fig. 2).

CD34-positive microvessels were detected in all the stages of GC, especially within the tumor parenchyma and intertumoral septa (Fig. 1). However, MVD differed remarkably according to GC stage (Fig. 1); the calculated mean MVD was 29.6 ± 20.9 vessels/field in EGC, whereas the mean MVD was 82.5 ± 35.75 vessels/field in AGC-M0, and 87.0 ± 30.87 vessels/field in AGC-M1 (ANOVA, $P < 0.001$). That is, the mean MVD was higher in tumor tissues isolated from AGC-M0 and AGC-M1 than in those isolated from EGC (Fig. 1), although there was no significant difference between AGC-M0 and AGC-M1 (Bonferroni multiple comparison, $P > 0.05$). These results imply that tumor angiogenesis starts from EGC and progresses actively from AGC-M0 before the establishment of macroscopic metastasis.

Interestingly, immunohistochemical staining showed that the calculated mean MVD increased with an increase in serum HMGB1 levels (Fig. 2a). Linear regression analysis validated the results of immunohistochemical staining; serum HMGB1 levels were closely correlated with MVD in GC tissue (linear regression, $R = 0.608$, $P = 0.01$; Fig. 2b). These results imply that extracellular HMGB1 contributes to tumor angiogenesis of GC.

***In vitro* evidence of HMGB1 involvement in tumor angiogenesis of GC.** To confirm the involvement of HMGB1 in tumor angiogenesis of GC *in vitro*, we carried out *in vitro* angiogenesis experiments using GC cell lines. First, we used the *in vitro* HUVEC migration assay. Figure 3(a) shows that the migration activity of HUVECs cultured in CM derived from rhHMGB1-treated N87 cells was higher than that of HUVECs cultured in CM derived from rhHMGB1-untreated N87 cells ($P = 0.004$). This effect of rhHMGB1 was dramatically reversed by addition of anti-HMGB1 Ab treatment ($P = 0.006$; Fig. 3a). Similar results were obtained from HUVECs cultured in CM derived from KATOIII cells ($P < 0.05$ for all; Fig. 3b). The HUVECs cultured in CM derived from HMGB1-TF N87 cells also showed increased migration activity compared to HUVECs cultured in CM derived from mock-TF cells ($P = 0.006$; Fig. 3c). This increase in migration activity by HMGB1-TF was reversed after siRNA-mediated inhibition of HMGB1 ($P = 0.008$; Fig. 3c). Similar results were obtained from HUVECs cultured in CM derived from KATOIII cells ($P < 0.05$ for all; Fig. 3d).

Next, we carried out the *in vitro* HUVEC capillary tube formation assay. Figure 4(a) shows that HUVECs cultured in CM derived from rhHMGB1-treated N87 and KATOIII cells markedly produced capillary-like tubes compared with HUVECs cultured in CM derived from rhHMGB1-untreated N87 and KATOIII cells ($P < 0.05$ for all). This effect of rhHMGB1 was reversed by addition of anti-HMGB1 Ab treatment, which was consistent with the results of the HUVEC migration assay in both N87 and KATOIII cells ($P < 0.05$ for all; Fig. 4a,c).

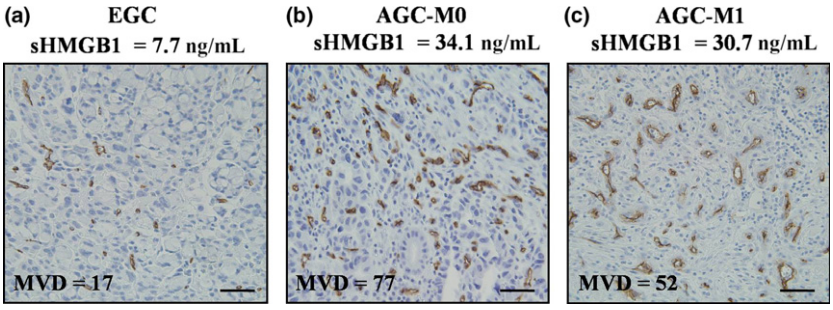

Fig. 1. CD34 immunohistochemical staining for microvessels in gastric cancer (GC) tumor sections (tumor angiogenesis) according to GC stage. (a) CD34-positive microvessels in early GC (EGC); serum high-mobility group box-1 (sHMGB1) level, 7.7 ng/mL. (b) CD34-positive microvessels in locally advanced GC (AGC-M0); sHMGB1 level, 34.1 ng/mL. (c) CD34-positive microvessels in highly advanced GC with metastasis (AGC-M1); sHMGB1 level, 30.7 ng/mL. Microvessel density (MVD) was determined as the average count of the five fields. Scale bar = 100 μm. Magnification, ×200 by light microscopy.

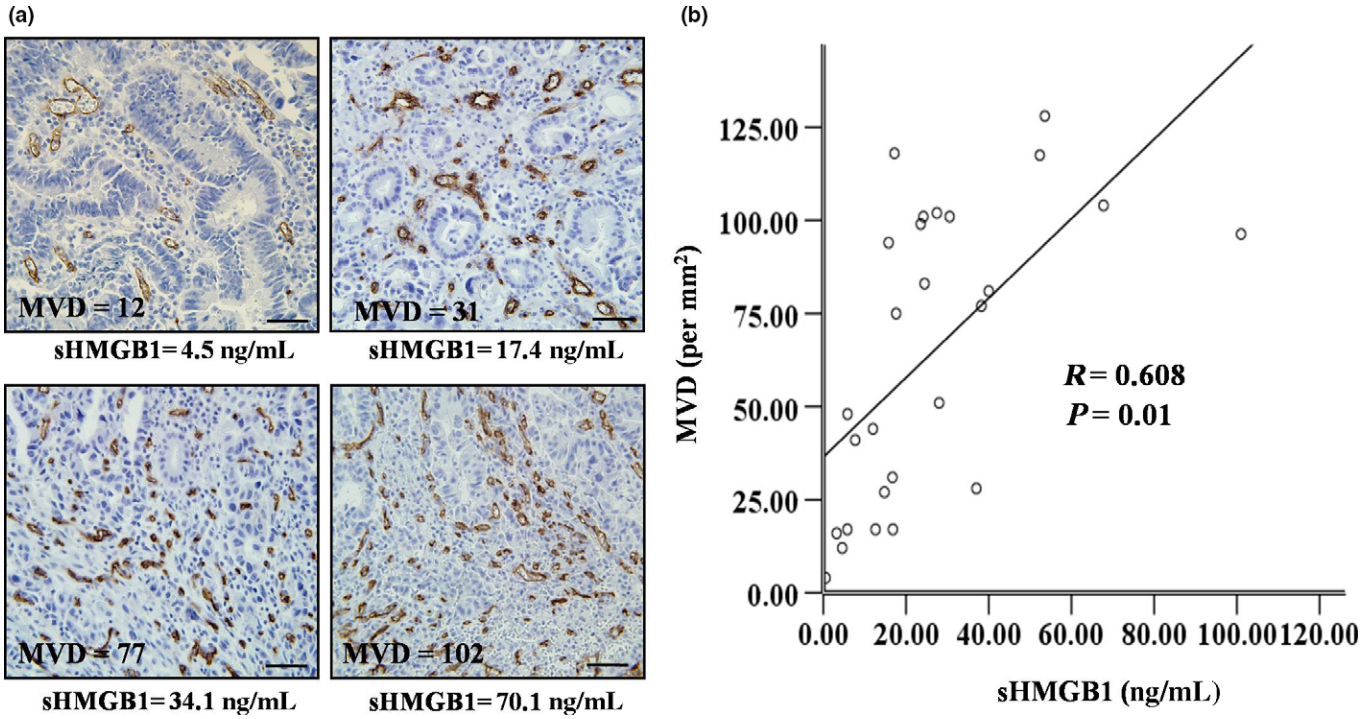

Fig. 2. Correlation of serum high-mobility group box-1 (sHMGB1) levels with microvessel density (MVD), as determined by quantifying CD34-positive microvessels in gastric cancer tumor sections. (a) Results of immunohistochemical staining for determining MVD according to sHMGB1 levels. Scale bar = 100 μm. (b) Linear regression analysis to determine the correlation of sHMGB1 levels with MVD. Magnification, ×200 by light microscopy.

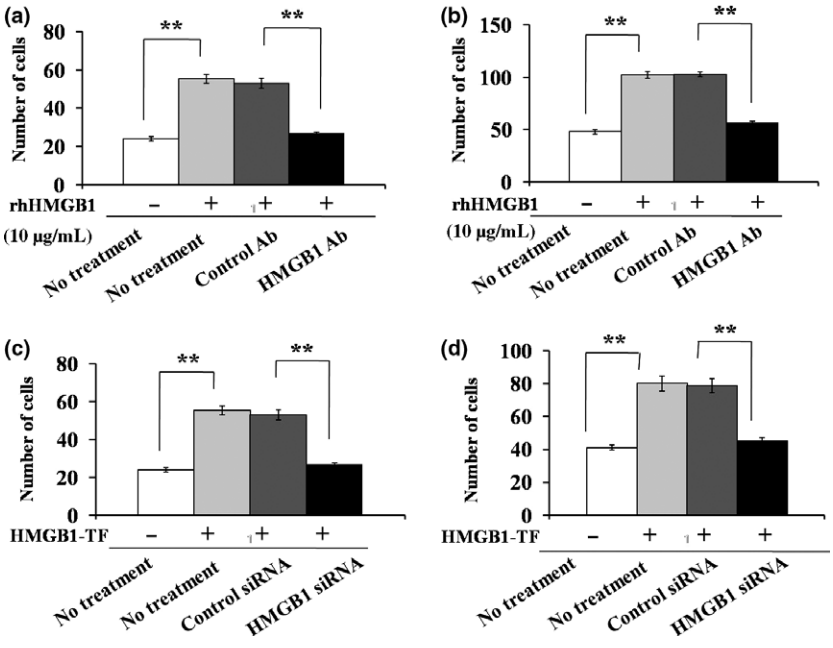

Fig. 3. *In vitro* angiogenesis assay in gastric cancer cell lines: HUVEC migration assay. (a) Migratory activity of HUVECs cultured in conditioned media (CM) derived from N87 cells without treatment, or treated with 10 μg/mL recombinant human high-mobility group box-1 (rhHMGB1), or 10 μg/mL rhHMGB1 plus 60 μg/mL anti-HMGB1 antibody for 24 h. (b) Migratory activity of HUVECs cultured in CM derived from untreated, rhHMGB1-treated, and rhHMGB1 plus anti-HMGB1 Ab-treated KATOIII cells for 24 h. (c) Migratory activity of HUVECs cultured in CM derived from mock-transfected (TF), HMGB1-TF, and HMGB1-TF plus HMGB1 siRNA-treated N87 cells for 24 h. (d) Migratory activity of HUVECs cultured in CM derived from mock-TF, HMGB1-TF, and HMGB1-TF plus HMGB1 siRNA-treated KATOIII cells for 24 h. Columns, mean number of HUVECs migrating through the inserts in three independent experiments, as observed using DP controller software with an IX71 microscope (magnification, ×100; Olympus Corporation). Staining, 2 μM calcein AM for 30 min. Bars, SE. *$P < 0.05$; **$P < 0.01$, Student's *t*-test.

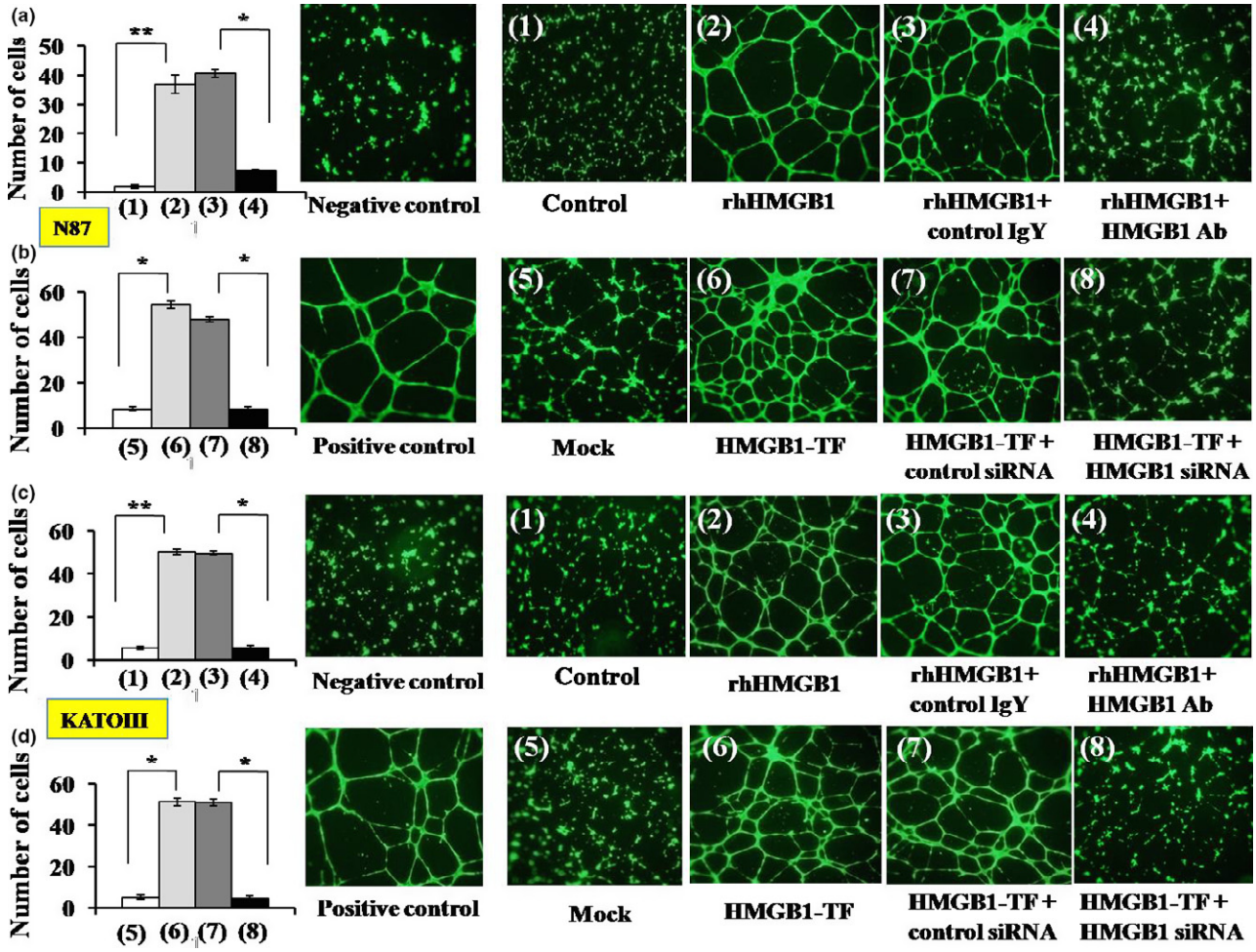

Fig. 4. *In vitro* angiogenesis assay in gastric cancer cell lines: HUVEC capillary tube formation assay. (a) Capillary tube formation activity of HUVECs cultured in conditioned medium (CM) derived from untreated, 10 μg/mL recombinant human high-mobility group box-1 (rhHMGB1)-treated, or 10 μg/mL rhHMGB1 plus 60 μg/mL anti-HMGB1 antibody (Ab)-treated N87 cells for 24 h. (b) Tube formation activity of HUVECs cultured in CM derived from mock-transfected (TF), HMGB1-TF, or HMGB1-TF plus HMGB1 siRNA-treated N87 cells for 24 h. (c) Tube formation activity of HUVECs cultured in CM derived from rhHMGB1-treated KATOIII cells. (d) Tube formation activity of HUVECs cultured in CM derived from HMGB1-TF KATOIII cells. Columns, mean counts of tube formation in three independent experiments (left). Bars, SE. $*P < 0.05$; $**P < 0.01$, Student's *t*-test. Images are obtained using DP controller software with an IX71 microscope (Olympus Corporation) (magnification, ×40; right). Staining, 2 μM calcein AM for 30 min.

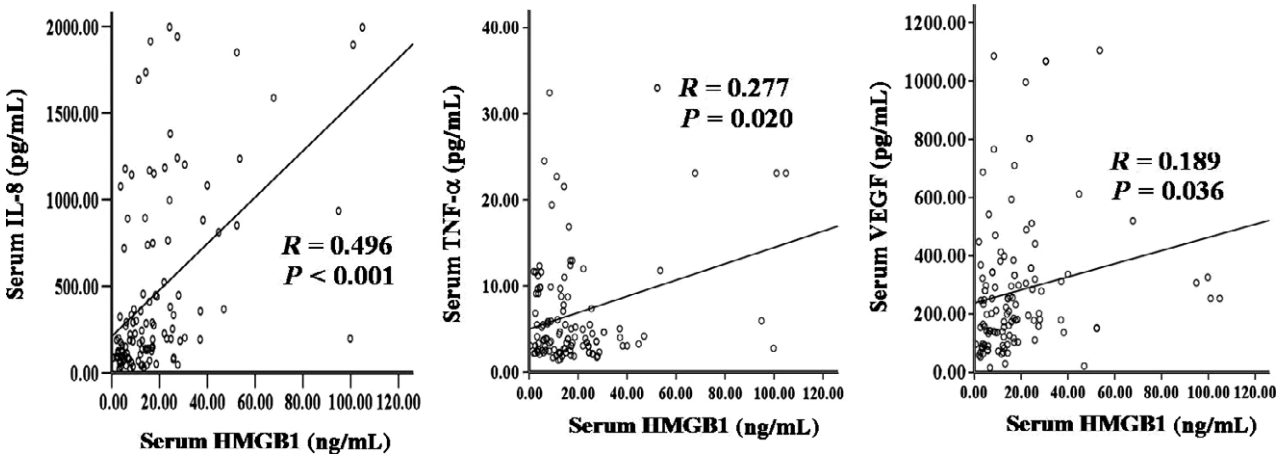

Fig. 5. Linear regression graphs presenting the correlation between serum high-mobility group box-1 (HMGB1) levels and serum cyokines/chemokines levels in gastric cancer. The graphs show the positive correlations of serum HMGB1 levels with serum interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and tumor necrosis factor-α (TNF-α) levels. Among them, serum IL-8 levels show the strongest correlation with serum HMGB1 levels.

Similarly, HUVECs cultured in CM derived from HMGB1-TF N87 and KATOIII cells showed higher capillary tube formation activities than HUVECs cultured in CM derived from mock-TF N87 and KATOIII cells ($P < 0.05$ for all; Fig. 4b,d). Overall, these results confirm that overexpressed extracellular HMGB1 contributes to tumor angiogenesis in GC cells *in vitro*.

Interleukin-8 mediates HMGB1-induced tumor angiogenesis. Many tumor-derived soluble factors are involved in the complex regulation of tumor angiogenesis. To determine which soluble factors mediate HMGB1-induced tumor angiogenesis, we evaluated serum levels of 20 cytokines/chemokines that are known to be involved in GC progression by chemiluminescent immunoassays using 120 GC serum samples. Of the examined cytokines/chemokines, serum levels of IL-8 ($R = 0.496$, $P < 0.001$), tumor necrosis factor-α ($R = 0.277$, $P = 0.020$), and VEGF ($R = 0.189$, $P = 0.036$) were significantly correlated with serum HMGB1 levels (linear regression; Fig. 5). Among these, IL-8 was the cytokine most significantly correlated with serum HMGB1.

Because serum IL-8 showed the strongest correlation with serum HMGB1 among the examined cytokines/chemokines ($P < 0.001$), and is a well-known pro-angiogenic factor in cancers,[23,24] IL-8 may be an essential tumor-derived soluble factor that mediates HMGB1-induced tumor angiogenesis in GC. To confirm our hypothesis, we examined whether the enhancement of rhHMGB1-induced or HMGB1-TF-induced HUVEC migration or tube formation activities can be abrogated by IL-8 inhibition. To prepare CM for this experiment, GC cells were cultured in RPMI-1640 supplemented with 1% FBS and rhHMGB1 (10 μg/mL) for 72 h with or without pretreatment of IL-8 siRNA, and the resultant culture medium was

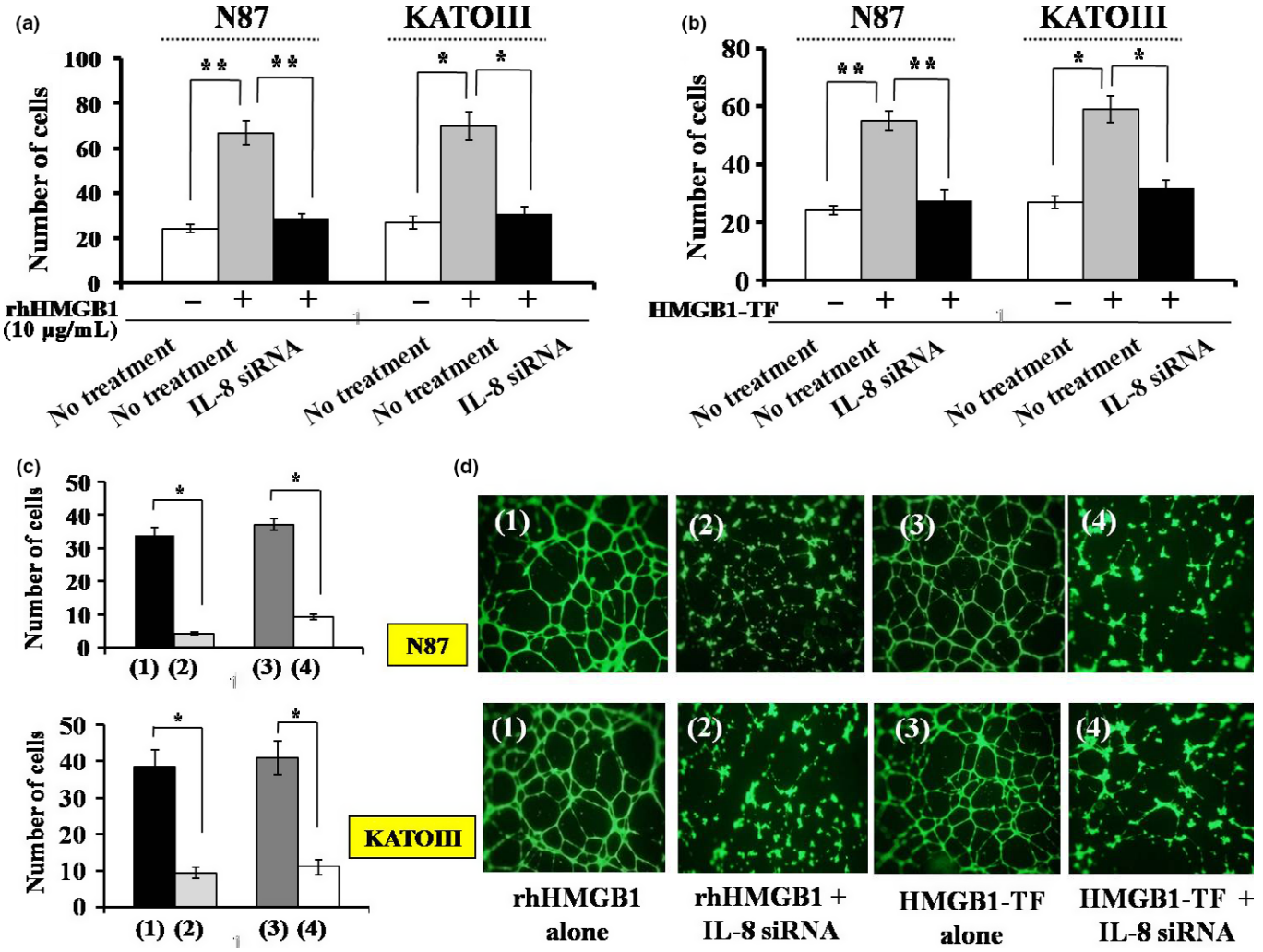

Fig. 6. *In vitro* angiogenesis assays showing that interleukin-8 (IL-8) mediates high-mobility group box-1 (HMGB1)-induced tumor angiogenesis in gastric cancer cells. (a, b) HUVEC migration assay. (a) Significant decline in the migratory activity of HUVECs cultured in CM derived from recombinant human HMGB1 (rhHMGB1; 10 μg/mL) plus IL-8 siRNA-treated N87 cells compared with that of HUVECs cultured in CM derived from cells treated with rhHMGB1 alone. (b) Significant decline in the migratory activity of HUVECs cultured in CM derived from HMGB1-transfected (HMGB1-TF) plus IL-8 siRNA-treated N87 cells compared with that of HUVECs cultured in CM derived from cells treated with HMGB1-TF alone. (c, d) HUVEC capillary tube formation assay. (c) Significant decline in tube formation activity of HUVECs cultured in CM derived from rhHMGB1 plus IL-8 siRNA-treated or HMGB1-TF plus IL-8 siRNA-treated N87 cells compared with that of HUVECs cultured in CM derived from rhHMGB1-treated or HMGB1-TF N87 cells (upper panel). Decline in tube formation activity of HUVECs cultured in CM derived from KATOIII cells in which IL-8 was inhibited by IL-8 siRNA (lower panel). (d) Images of capillary tube formation of HUVECs obtained using DP controller software with an IX71 microscope (magnification, ×40; Olympus Corporation). Columns, mean of three experiments. Bars, SE. *$P < 0.05$; **$P < 0.01$, Student's *t*-test.

collected. The collected medium was centrifuged, obtained as the supernatant, and used as CM. Similarly, HMGB1-TF GC cells were cultured in RPMI-1640 supplemented with 1% FBS for 72 h with or without pretreatment of IL-8 siRNA, and the resultant culture medium was collected for CM preparation.

As expected, the migration activity of HUVECs cultured in CM derived from GC cells treated with both rhHMGB1 and IL-8 siRNA was remarkably reduced compared with that of HUVECs cultured in CM derived from GC cells treated with rhHMGB1 alone (Fig. 6a). Similarily, the enhancement of migration activity of HUVECs by HMGB1-TF was abrogated by pretreatment of IL-8 siRNA (Fig. 6b).

The enhancement of capillary-like tube formation activity of HUVECs by rhHMGB1-treatment or HMGB1-TF was also abrogated by pretreatment with IL-8 siRNA (Fig. 6c,d). Taken together, our findings indicate that IL-8 mediates extracellular HMGB1-induced tumor angiogenesis in GC.

Discussion

High-mobility group box-1, originally known as a nuclear protein, acts as a crucial pro-inflammatory cytokine in its secretory form.[15,16] Recent studies have shown that overexpressed extracellular HMGB1 contributes to cancer carinogenesis and metastasis by promoting apoptotic evasion, mediating tumor-associated inflammation, and promoting tumor cell migration and angiogenesis.[15–18] However, the exact role of overexpressed extracellular HMGB1 in cancer progression is still under investigation.

Because HMGB1 belongs to the high-mobility group superfamily, this protein is expected to contribute to angiogenesis in various situations, including cancers. Many recent studies have shown that HMGB1 is involved in tumor angiogenesis in several cancers.[17,18] Previously, we showed that high levels of serum HMGB1 are closely correlated with GC progression in GC patients from the pre-invasive stage.[20] We also reported that overexpressed extracellular HMGB1 induced EMT, a critical process for metastasis initiation, under overxpressed IL-8 mediation in GC.[25] Interleukin-8 is also a well-known angiogenic and tumor aggravation factor in many cancers, including GC.[23,24,26,27] Because both HMGB1 and IL-8 contribute to tumor angiogensis in many cancers, and angiogenesis may be necessary at any stage during cancer progression,[28] it is possible that interaction between HMGB1 and IL-8 may contribute to GC progression by inducing tumor angiogenesis as well as inducing EMT[25] from the relatively early steps of metastasis. Therefore, we investigated whether overexpressed extracellular HMGB1 contributes to tumor angiogenesis in GC with IL-8 mediation in the present study.

To prove our hypothesis, we first showed that overexpressed extracellular HMGB1 contributes to tumor angiogenesis in GC by showing a positive correlation between serum HMGB1 levels and MVD in GC tissues clinically (Fig. 2), and by showing the enhancement of HUVEC migration and tube formation activities through culturing HUVECs in CM derived from rhHMGB1-treated or HMGB1-TF GC cells *in vitro* (Figs. 3,4). These findings indicate that extracellular HMGB1 overexpression contributes to tumor angiogenesis in GC. Next, we analyzed 20 GC-related cytokines/chemokines using 120 GC serum samples to determine soluble factors involved in HMGB1-induced tumor angiogenesis. We found that IL-8 was the cytokine/chemokine most significantly correlated with serum HMGB1 ($R = 0.496$, $P < 0.001$; Fig. 5). This agreed with results reported in our previous study,[25] in which independent GC serum samples and a different cytokine panel were used. Finally, we found that IL-8 inhibition suppressed the overexpressed HMGB1-induced increase in HUVEC migration and tube formation activities (Fig. 6). Collectively, we showed that overexpressed extracellular HMGB1 can induce tumor angiogenesis in GC, and IL-8 might mediate this process.

Interaction between VEGF-A and the VEGF receptor-1 or -2 axis is a key process for blood vessel growth.[4] In the current study, we observed that VEGF levels were also correlated with serum HMGB1 levels. This result may suggest that VEGF is also involved in HMGB1-induced tumor angiogenesis.

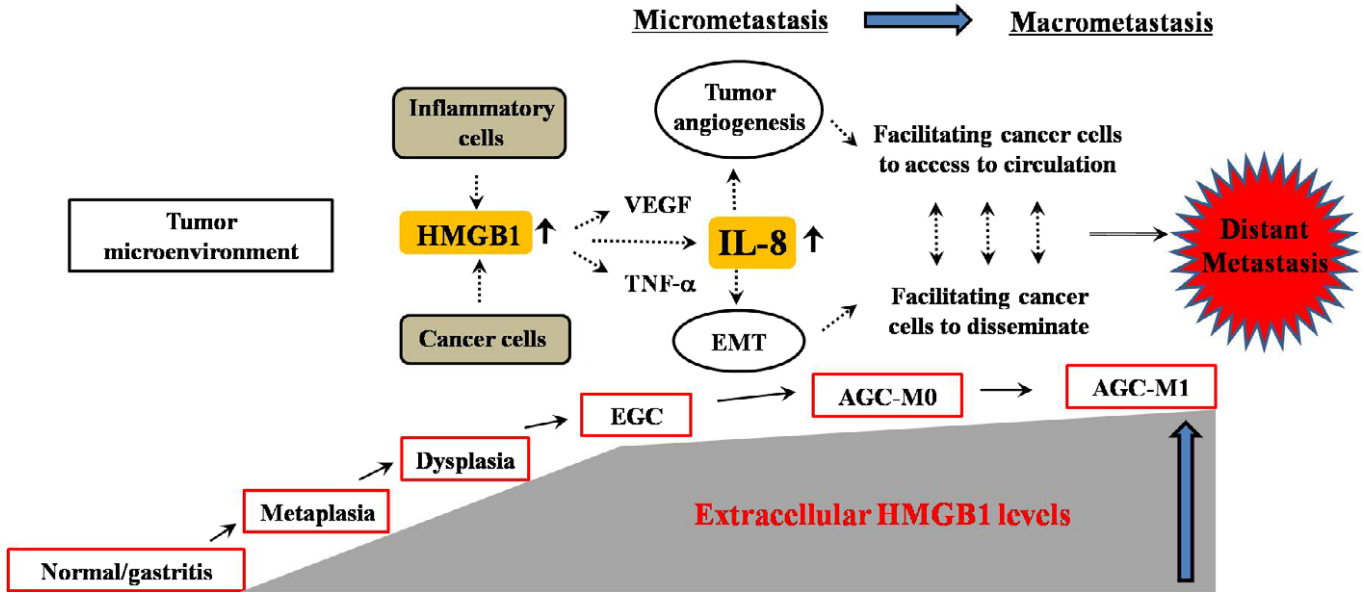

Fig. 7. Schematic figure of a hypothetic model of interaction between extracellular high-mobility group box-1 (HMGB1) and interleukin-8 (IL-8) during gastric cancer (GC) progression. This interaction may contribute to GC progression at a relatively early stage of GC metastasis, providing a mechanistic link between tumor angiogenesis and epithelial–mesenchymal transition (EMT) through HMGB1 and IL-8 interaction. AGC-M0, locally advanced GC without metastasis. AGC-M1, highly advanced GC with metastasis. EGC, early GC; TNF-α, tumor necrosis factor-α.

However, we did not evaluate the effects of VEGF in the current study because the degree of correlation of this cytokine with serum HMGB1 was not significantly higher ($R = 0.189$, $P = 0.036$) than that of IL-8 (Fig. 5). Thus, VEGF may not be a major soluble mediator involved in the HMGB1-induced tumor angiogenesis. Instead, it is possible that VEGF may be involved in HMGB1-induced tumor angiogenesis indirectly through connection with IL-8, similar to the results reported in several previous studies.[23,28] However, further additional studies are needed to confirm this.

Another previous study co-cultured HUVECs with GC cells directly to access *in vitro* angiogenesis.[23] However, we cultured HUVECs in CM derived from rhHMGB1-treated or HMGB1-TF GC cells instead of co-culturing with GC cells to exclude interference of factors other than overexpressed HMGB1.

For *in vitro* experiments, we used two different histological types of GC cell lines, namely, N87, a well-differentiated adenocarcinoma cell line, and KATOIII, a signet-ring cell carcinoma cell line, because these two cell lines have different clinicopathological features. However, the effect of overexpressed extracellular HMGB1 on tumor angiogenesis was not significantly different between the two cell lines (Figs. 3,4,6).

In summary, the results of the present study indicate that overexpressed extracellular HMGB1 promotes tumor angiogenesis under IL-8 mediation in GC. To our knowledge, this is the first study to show that HMGB1 contributes to tumor angiogenesis in GC and this process is associated with IL-8. In addition, the results of our previous[25] and current study suggest a model of interaction in which extracellular HMGB1 and IL-8 may contribute to GC progression, providing a mechanistic link between tumor angiogenesis and EMT at a relatively early stage of GC metastasis (Fig. 7). That is, high levels of HMGB1 may increase the recurrence and metastasis of GC by facilitating easy dissemination of cancer cells in circulation through both EMT induction and tumor angiogenesis, and IL-8 is the major HMGB1-associated soluble mediator that participates in these two processes simultaneously during GC progression (Fig. 7).

In conclusion, results of the present study suggest the potential efficacy of combined targeting of HMGB1 and IL-8 for controlling tumor angiogenesis and ultimately improving the poor prognosis of patients with GC. We believe that our findings provide important clues on the mechanisms underlying the high rate of recurrence and metastasis of GC, and could help in developing a new promising therapeutic strategy for GC.

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Disclosure Statement

The authors have no conflict of interest.

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