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Original Article

The significance of tumor cells-derived MFG-E8 in tumor growth of angiosarcoma

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ABSTRACT

Background: Previous studies have indicated that MFG-E8 enhances tumor cell survival, invasion and angiogenesis. However, the role of MFG-E8 in angiosarcoma (AS) has not been clarified.
Objective: Objective was to elucidate the mechanism of the regulation by MFG-E8 in AS and the association between MFG-E8 and clinicopathological features of AS.
Methods: The effects of the depletion of MFG-E8 by siRNA on tube formation, migration and proliferation in murine AS cells were examined. The effect of administration of anti-MFG-E8 antibody (Ab) on tumor growth of AS in mice was examined. The associations of MFG-E8 expression and clinicopathological features of human AS were assessed.
Results: The expressions of MFG-E8 in murine and human AS cells were significantly higher than those in melanoma cells, macrophages and endothelial cells. Depletion of MFG-E8 in murine AS cells by siRNA significantly inhibited the formation of capillary-like structures and migration, but not proliferation. Administration of anti-MFG-E8 Ab significantly inhibited tumor growth and decreased the number of tumor-associated macrophages (TAMs) in AS tumors. Tumor size and the number of TAMs in human AS with high expression of MFG-E8 were significantly increased compared to those of AS with low expression of MFG-E8. Progression-free survival and overall survival time of the patients of AS with high expression of MFG-E8 were significantly shorter than those of AS with low expression of MFG-E8.
Conclusions: AS-derived MFG-E8 might enhance tumor growth *via* angiogenesis and the induction of TAMs in autocrine/paracrine manner, and administration of anti-MFG-E8 Ab could be a therapeutic potential for AS.

1. Introduction

Angiosarcoma (AS) develops in various soft tissues and organs, but the most frequently affected site is the skin, commonly occurring in the scalp of elderly people and typically presenting as an enlarging bruise-like purpura [1–5]. In the most of patients, AS is relatively large at the time of diagnosis because of a significant delay in diagnosis. There are some treatment options, however, an effective treatment has not been established yet, resulting in the poor prognosis.

Milk fat globule-associated protein with EGF- and factor-8 like domains (MFG-E8) is a secreted glycoprotein, and murine MFG-E8 is composed of two N-terminal EGF-like domains and two C-terminal discoidin-like domains that are homologous to blood

Abbreviations: AS, angiosarcoma; Ab, antibody; TAMs, tumor-associated macrophages; MFG-E8, milk fat gelobule-associated protein with EGF- and factor-8 like domains; MSCs, mesenchymal stem cells; PDGFR, platelet-derived growth factor receptor; VEGF, vascular endothelial growth factor; ET-1, endothelin-1; WB, Western blotting; ECs, endothelial cells; OS, overall survival; PFS, progression-free survival; Treg, regulatory T cells.

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coagulation factor 5 and 8 [6,7]. Murine MFG-E8 has two isoforms, long and short forms, based on the presence of the proline/threonine rich repeats (P/T) [8]. Human MFG-E8 is composed of one N-terminal EGF-like domain and two C-terminal discoidin-like domains [9]. One EGF-like domain contains the arginine-glycine-aspartic acid (RGD) domain that binds to integrin αvβ3/β5, and the C-domains bind to phosphatidylserine and collagen type I [10,11]. MFG-E8 accelerates phagocytosis of apoptotic cells by bridging phosphatidylserine in apoptotic cells and integrin αvβ3/β5 in phagocytes, resulting in the suppression of inflammatory responses [12–14]. We previously found that pericytes and mesenchymal stem cells (MSCs) secrete large amount of MFG-E8, and pericyte- and MSCs-derived MFG-E8 promotes platelet-derived growth factor receptor (PDGFR) signaling in pericytes/MSCs via binding to integrin αvβ3/β5, resulting in the promoting angiogenesis in melanoma tumors [15–18]. In addition, we found that MFG-E8 enhanced the number of tumor associated macrophages (TAMs) in melanoma tumors *in vivo*, and that MFG-E8 from MSCs enhanced MSCs-induced M2 macrophage polarization *in vitro* [17,18]. Furthermore, we demonstrated that MFG-E8 enhanced angiogenesis and the infiltration of M2 macrophages, but reduced inflammation in the wound, suggesting that MFG-E8 might have a potential as a therapeutic option for the treatment of diabetic or decubitus ulcers [19–21].

A protumoral function of MFGE8 has been documented for severaral types of human cancers, including oral, esophageal, colorectal, breast, bladder, ovarian and prostate carcinoma and melanoma [22–30]. It has been reported that MFG-E8 expression increased significantly in colorectal cancer compared with normal mucosa tissues, and high MFG-E8 expression was correlated with lymph node metastasis and shortened overall survivals, suggesting that MFG-E8 could be a prognostic marker for colorectal cancer and overexpression of MFG-E8 might be involved in lymph node metastasis and angiogenesis of colorectal cancer [24,25]. It has been reported that anti-MFG-E8 monoclonal antibody (Ab) inhibited adhesion, migration and survival of MFGE8-expressing ovarian carcinoma *in vitro* [27]. However, the mechanism of the regulation of tumor growth of AS by MFG-E8, and the association of MFG-E8 and clinicopathological features of AS has not been clarified. In this study, we examined MFG-E8 expression in murine/human AS cell lines, and the effects of the depletion of MFG-E8 by siRNA on tube formation, migration and proliferation in murine AS cells. We further examined the effect of administration of anti-MFG-E8 Ab on tumor growth of AS in mice. The associations of MFG-E8 expression and clinicopathological features of human AS were also assessed.

2. Materials and methods

2.1. Patients and clinical assessments

We analyzed 50 Japanese patients with AS (16 female and 34 male; age 76.7 ± 1.1 years). The study was approved by the institutional review board and the local research ethics committee of Gunma University. This study was conducted according to the Declaration of Helsinki principles. The detail is explained in Supplementary materials and methods.

2.2. Cell culture

Mouse AS cell line (SVR), mouse melanoma cell line (B16-F10), mouse monocyte/macrophage cell line (RAW 264.7) and human umbilical vein endothelial cell (HUVEC) were obtained directly from the ATCC (Manassas, VA). Mouse MSC was obtained and incubated as described previously [18,21,31]. Human cutaneous AS cell line, HAMON was incubated as previously described [32]. Human AS cell line was established from surgically resected tissue. HAMON expresses CD31, vascular endothelial growth factor (VEGF) and VEGF receptor 2. The implanted HAMON into immunodeficient mice develops tumors [32]. The detail is explained in Supplementary materials and methods.

2.3. Real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated using RNeasy mini kits (Qiagen, Valencia, CA) and was subjected to reverse transcription with use of a GoScript Reverse Transcription system (Promega, Madison, WI). Real-time quantitative reverse transcription PCR (RT-qPCR) was performed using a SYBR Green system (Thermo Fisher Scientific, Waltham, MA) with ABI 7300 real-time PCR instrumentation (Applied Biosytems, Carlsbad, CA). Normalization and fold changes were calculated using the comparative CT method.

2.4. MFG-E8 knockdown experiments

To inhibit the expression of MFG-E8, siRNA specific for mouse MFG-E8 mRNA was designed and purchased with QIAGEN. SVR AS cells (5×10^5 cells) were transfected with 20 nM MFG-E8 siRNA or AllStars negative control siRNA (QIAGEN) using Lipofectamine RNAiMAX Reagent (Invitrogen). After 48 h, MFG-E8, VEGF, endothelin-1 (ET-1), angiopoietin and 18 s mRNA levels were assessed by quantitative RT-PCR and MFG-E8 protein level was assessed by Western blotting (WB). WB analyses were performed according to the previously described protocols [16,33].

2.5. Proliferation assay

Cell proliferation was measured using the MTS assay. SVR cells were transfected with MFG-E8 siRNA or control siRNA. Twenty-one hours after transfection, cells were treated with trypsin-EDTA and plated at a density of 5000 cells per well with 0.5% FCS containing medium in 96 well plates. After 48 h incubation at 37 °C, 20 μl of CellTiter 96 AQueous One Solution Reagent (Promega) was added. After an additional incubation at 37 °C for 2 h, the absorbance at 490 nm was measured using an ELISA plate reader.

2.6. Migration assay

Cell migration was assessed in an *in vitro* wound healing ("scratch") assay. In these experiments, SVR cells transfected with MFG-E8 siRNA or control siRNA were incubated in 0.5% FCS-containing medium overnight, confluent cell monolayer was scratched with a pipette tip and displaced cells were removed with washing. "Wounded" monolayers were incubated for 18 h, images of wounded areas were acquired at identical locations at the beginning and the end of the assay and the areas of residual "wounds" were determined using Image J.

2.7. Capillary-like structure formation assay

The capillary-like structure formation assay was performed as described previously [34,35]. At 24 h after siRNA transfection, 1.0×10^5 SVR cells were cultured in solidified Matrigel (200 μl/well in 24-well plates) (BD Biosciences, San Jose, CA) and were incubated at 37 °C. After 3 h, six random fields were chosen in each well, and the total length of capillary-like structures was quantified using Image J software. To examine the effects of MFG-E8 siRNA, MFG-E8 mRNA levels were also assessed by RT-qPCR immediately after the tube formation assay.

2.8. AS xenograft model

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Eight week-old C57BL/6 mice were used for all

experiments. Mice were maintained at the Institute of Experimental Animal Research of Gunma University Graduate School of Medicine under specific pathogen-free conditions. All experiments were approved by the Gunma University Animal Care and Experimentation Committee (#15-059), and carried out in accordance with the approved guidelines. SVR cells (1×10^7cells) were implanted subcutaneously into the flanks of C57BL/6 mice at once (day 0). Tumor sizes (width × length; mm^2) were determined with calipers every day until the tumor disappeared. To analyze the effect of anti-MFG-E8 Ab on tumor growth, 30 ng armenian hamster anti-mouse MFG-E8 Ab (MBL, Nagoya, Japan) per 100 μl PBS or the same concentration of armenian hamster IgG isotype control (Invitrogen) was injected intraperitoneally at day 0 and day 1.

2.9. Immunohistochemistry and immunofluorescence staining

The detail is explained in Supplementary materials and methods. MFG-E8 staining in human AS tumors was assessed on the basis of the proportion of the stained cells and immunostaining intensity. The proportion of MFG-E8 positive cells was graded as 0 (negative), 1 (<1% positive), 2 (1–5% positive), 3 (>5% positive), and staining intensity was graded as 0 (negative), 1 (weak positive), 2 (strong positive) (Fig. 4 A), according to previous report [22]. The score for the proportion of stained cells and staining intensity were multiplied to provide the MFG-E8 expression score, 0–6. MFG-E8 expression score in human AS tumors was quantified by C. F. and S. M.

P values were calculated by a Student's *t*-test or one-way ANOVA followed by Bonferroni's post test for multiple comparisons. Error bars represent standard errors of the mean, and numbers of experiments (n) are as indicated.

3. Results

3.1. Murine and human AS cells and AS tumors in mice produced a substantial amount of MFG-E8

To examine the expression of MFG-E8 in AS cells, we first compared the expression of MFG-E8 mRNA in murine AS cell line (SVR), murine macrophages (RAW 264.7), murine melanoma cells (B16F10) and murine bone marrow-derived MSCs. We found that the expression of MFG-E8 mRNA of SVR was significantly higher than that of macrophages and melanoma cells (Fig. 1A), while the expression of MFG-E8 mRNA of MSCs was higher than that of SVR (Fig. 1A). Next, we compared the expression of mRNA levels of MFG-E8 in human endothelial cells (ECs) (HUVEC) and human AS cells (HAMON). The expression of MFG-E8 mRNA of human AS cells was significantly higher than that of ECs (Fig. 1B). We next assessed the expression of MFG-E8 in AS tumors in mice. SVR cells were implanted subcutaneously into the flanks of mice, and AS tumors were resected 3 days after implantation. H&E staining demonstrated that the tumor consisted of various-sized immature vessels, compatible with the histological characteristics of AS (Fig. 1C). We confirmed that the AS tumor cells expressed CD31 by immunohistochemical staining (Fig. 1C). Furthermore, MFG-E8 was stained in AS tumor cells and interstitial area (Fig. 1C). These results suggest that murine and human AS cells and AS tumors in mice might produce a substantial amount of MFG-E8.

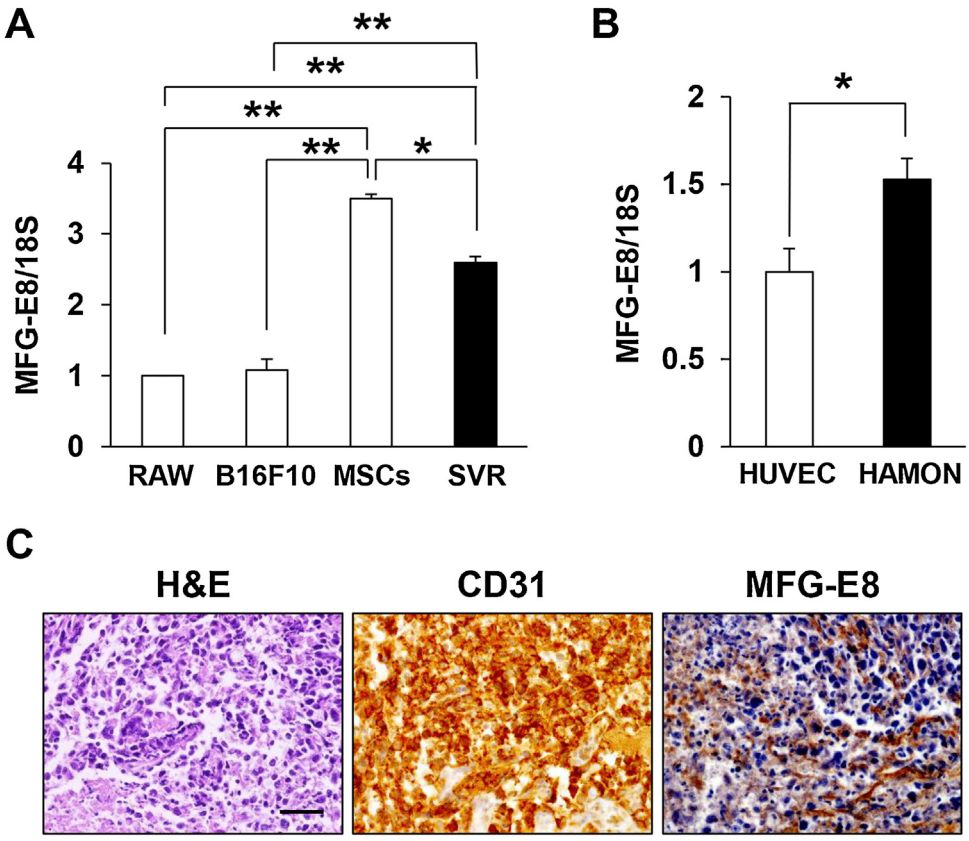


Fig. 1. Murine and human AS cells and AS tumors in mice produced a substantial amount of MFG-E8. (A) The expressions of MFG-E8 mRNA in murine AS cell line (SVR), murine macrophages (RAW 264.7), murine melanoma cells (B16F10) and murine bone marrow-derived MSCs. mRNA level in RAW was assigned a value of 1. n = 3. (B) The expressions of MFG-E8 mRNA in human ECs (HUVEC) and human AS cells (HAMON). mRNA level in HUVEC was assigned a value of 1. n = 3. (C) Representative images of the H&E, CD31 and MFG-E8 staining of the section of AS tumors in mice. Scale bar = 50 μm. All values represent mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$.

3.2. Depletion of MFG-E8 significantly inhibited migration and the formation of capillary-like structures of AS cells

Next, we examined the effects of siRNA depletion of MFG-E8 in murine AS cells (SVR). The expression of MFG-E8 mRNA in AS cells treated with MFG-E8 siRNA was reduced by 70% compared with that in AS cells treated with control siRNA (Fig. 2A). The protein level of MFG-E8 in AS cells was reduced by MFG-E8 siRNA by immunoblotting (Fig. 2B). The mRNA levels of angiogenic factors, such as VEGF, ET-1 and angiopoietin in control or MFG-E8 siRNA-treated AS cells were not significantly changed (Fig. 2C) (VEGF: $P = 0.3$, ET-1: $P = 0.1$, Angiopoietin: $P = 0.75$). In addition, cell proliferation of murine AS cells was not changed by the depletion of MFG-E8 by siRNA (Fig. 2D). Furthermore, we examined the effects of siRNA depletion of MFG-E8 in human AS cells (HAMON). The expression of MFG-E8 mRNA in human AS cells treated with MFG-E8 siRNA was reduced by 90% compared with that in AS cells treated with control siRNA (Supplementary Fig. 1A). The mRNA levels of VEGF, ET-1 and angiopoietin in control or MFG-E8 siRNA-treated human AS cells were not significantly changed (Supplementary Fig. 1B) (VEGF: $P = 0.58$, ET-1: $P = 0.8$, Angiopoietin: $P = 0.12$). In addition, cell proliferation of human AS cells was not changed by the depletion of MFG-E8 by siRNA (Supplementary Fig. 1C).

We next examined the role of MFG-E8 in cell migration of murine AS cells using a scratch assay. Migration of murine AS cells was significantly inhibited by the depletion of MFG-E8 by siRNA (Fig. 2E). We further examined the effects of MFG-E8 depletion on the formation of capillary-like structure using control or MFG-E8 siRNA-treated murine AS cells. Depletion of MFG-E8 in murine AS cells significantly reduced formation of capillary-like structure (Fig. 2F). These results suggest that AS cells-derived MFG-E8 might enhance cell migration and capillary formation in an autocrine and paracrine manner, resulting in the enhancement of angiogenesis.

3.3. Administration of anti-MFG-E8 antibody significantly inhibited the infiltration of TAMs and tumor growth of AS in vivo

We further examined the effect of anti-MFG-E8 Ab injection on the growth of AS tumors in mice. The implanted AS tumors grew into the dark reddish, easily bleeding tumors in 3–4 days after implantation. The size of the tumor peaked at 3–4 days after implantation, then it gradually shrunk and disappeared spontaneously about 20 days after implantation (Fig. 3A). We identified that the administration of anti-MFG-E8 Ab significantly inhibited the size of AS tumors in mice compared to control IgG from 1 to 9 days after implantation (Fig. 3A). Next, the number of $CD68^+$ macrophages and $CD163^+$ M2-type TAMs in AS tumors were assessed. We determined that the number of $CD68^+$ macrophages tended to decrease, and the number of $CD163^+$ TAMs in AS tumors was significantly decreased by the administration of anti-MFG-E8 Ab

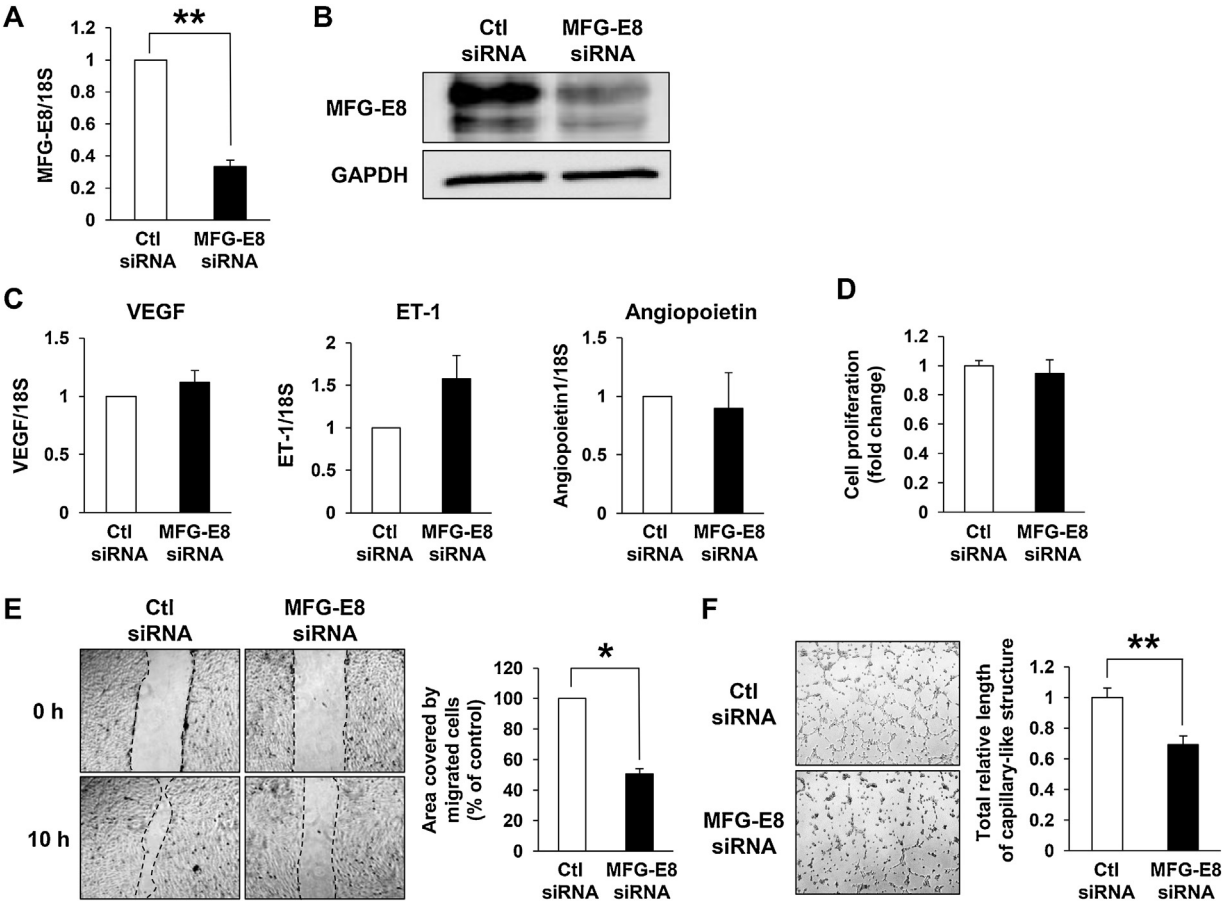


Fig. 2. Depletion of MFG-E8 significantly inhibited migration and the formation of capillary-like structures of murine AS cells. (A, B) The expressions of mRNA and protein levels of MFG-E8 in AS cells treated with control or MFG-E8 siRNA. n = 3. (C) The expressions of VEGF, ET-1 and angiopoietin in AS cells treated with control or MFG-E8 siRNA. n = 3. (D) The proliferation of AS cells treated with control or MFG-E8 siRNA. n = 3. (E) Cell migration of AS cells treated with control or MFG-E8 siRNA. Quantification of area covered by migrated cells in three random microscopic fields in three independent experiments was performed. (F) The formation of capillary-like structure by AS cells treated with control or MFG-E8 siRNA. Quantification of total relative length of capillary-like structures in six random microscopic fields in three independent experiments was performed. mRNA level, proliferation or the total length of capillary-like structure in control siRNA was assigned a value of 1. All values represent mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$.

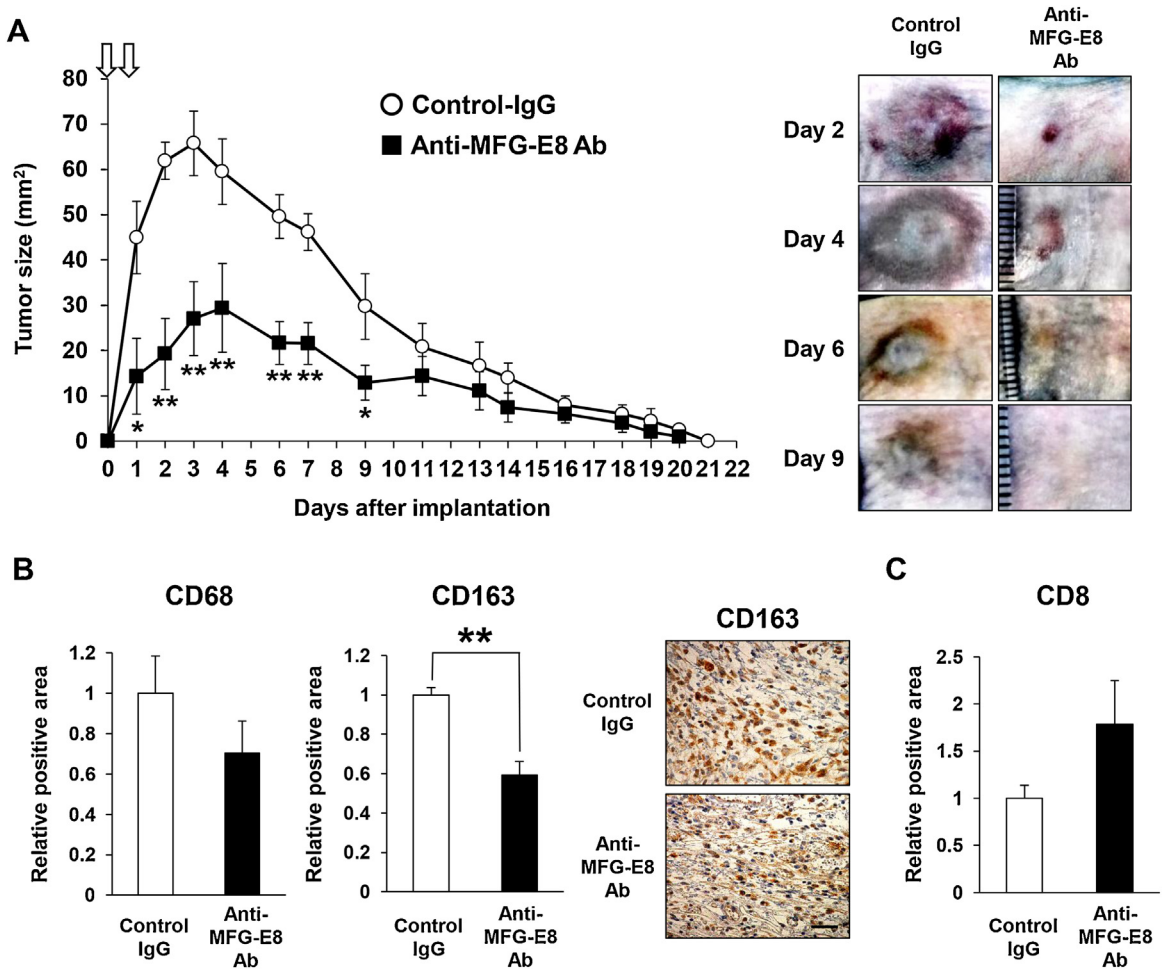


Fig. 3. Administration of anti-MFG-E8 antibody significantly inhibited the infiltration of TAMs and tumor growth of AS *in vivo*. (A) Tumor growth of AS tumors in mice injected with control-IgG or anti-MFG-E8 antibody. n = 5 mice in each group. Photographs of AS tumors in control-IgG or anti-MFG-E8 antibody injected mice at 2, 4, 6 and 9 days after implantation. (B) Numbers of $CD68^+$ macrophages and $CD163^+$ TAMs in AS tumors treated with control-IgG or anti-MFG-E8 antibody. Scale bar = 50 μm. n = 5 mice in each group. (C) Number of $CD8^+$ T cells in AS tumors treated with control-IgG or anti-MFG-E8 antibody. n = 5 mice in each group. All values represent mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$.

(Fig. 3B) (CD68: $P = 0.26$, CD163: $P < 0.01$). The number of $CD8^+$ T cells in AS tumors in mice treated with anti-MFG-E8 Ab tended to increase in comparison with that in mice treated with control IgG, however, the difference was not significant (Fig. 3C) (CD8: $P = 0.19$). These results suggest that the administration of anti-MFG-E8 Ab might inhibit the infiltration of TAMs and tumor growth of AS *in vivo*.

3.4. High MFG-E8 expression in AS tumor might be associated with poor prognosis in patients with AS

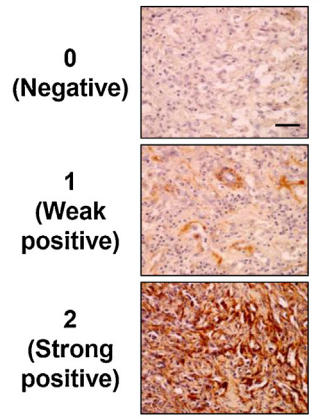
We finally examined the expression of MFG-E8 by immunohistochemical staining in 50 human AS tumors and compared the clinicopathological features between high and low expressions of MFG-E8 in human AS tumors. MFG-E8 staining in AS tumor was assessed on the basis of the proportion of the stained cells and immunostaining intensity. Staining intensity score of 0, 1 and 2 were given when MFG-E8 staining was negative, weak positive and strong positive, respectively (Fig. 4A). The score for the proportion of stained cells and staining intensity were multiplied to provide total score, 0–6 [22]. As the result of MFG-E8 staining scoring, 12 cases were graded as score 0 (24%), 8 as score 1 (16%), 11 as score 2 (22%), 3 as score 3 (6%), 7 as score 4 (14%), and 9 as score 6 (18%). Based on these scores, 50 cases were grouped into two categories; low expression (scores of ≤1, 20 cases, 40%) and high expression (a score of ≥2, 30 cases, 60%). Clinical features of human AS with high and low expressions of MFG-E8 were shown in Fig. 4B. The size of high MFG-E8 expression tumor was significantly bigger than that of low MFG-E8 expression tumor. Furthermore, overall survival (OS) and progression-free survival (PFS) time of the patients with high MFG-E8 expression tumor were significantly shorter than those of the patients with low MFG-E8 expression tumor. There were no significant differences in age, sex, lymph node metastasis, distant metastasis and ulceration between two groups.

Furthermore, we assessed the number of infiltrated TAMs in human AS. The number of $CD68^+$ cells in low MFG-E8 expression tumor was tendency to be reduced compared to those in high MFG-E8 expression tumor, however, there was no significant difference between two groups (Fig. 4C). While, the number of $CD163^+$ TAMs was significantly reduced in low MFG-E8 expression tumors (Fig. 4C). These results suggest that high MFG-E8 expression in AS tumor might be associated with the increased tumor growth and the decreased infiltration of TAMs, as well as poor prognosis in patients with AS.

4. Discussion

We previously reported that pericytes and/or pericyte precursors were predominant source of MFG-E8 in melanoma tumors,

A



B

	MFG-E8 expression		*P* value
	High (60%, n=30)	Low (40%, n=20)	
Age	77.7 ± 1.4	75.1 ± 1.9	0.804
Sex			
Female	10	6	0.255
Male	20	14	
Size (mm)	70.5 ± 9.3	40.1 ± 3.9	0.008**
Lymph node metastasis	14% (3/21)	10% (2/20)	0.675
Distant metastasis	20% (5/25)	20% (4/20)	1
Survival time (months)	15.4 ± 1.6	28.9 ± 5.0	0.028*
Progression-free survival (months)	10.2 ± 1.6	19.2 ± 3.7	0.039*
Ulceration	68% (17/25)	69% (11/16)	0.96

C

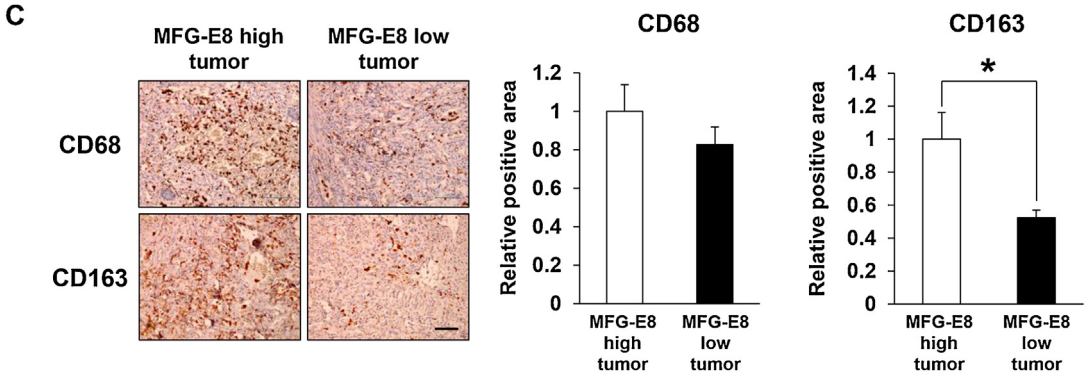


Fig. 4. High MFG-E8 expression in AS tumor might be associated with poor prognosis in patients with AS. (A) Representative images of the score of staining intensity of MFG-E8 in human AS tumors; 0: negative, 1: weak positive and 2: strong positive. Scale bar = 50 μm. (B) Clinical features of human AS with high and low expressions of MFG-E8. (C) Number of $CD68^+$ macrophages and $CD163^+$ TAMs in human AS tumors with high and low expressions of MFG-E8. Scale bar =100 μm. All values represent mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$.

and pericytes/pericyte precursor-derived MFG-E8 enhanced angiogenesis in melanoma tumors [15,16]. In addition, we previously identified that BM-MSCs produced large amounts of MFG-E8 [18,21]. In this study, we demonstrated that murine AS cells produce as much MFG-E8 as mouse MSCs, and that murine and human AS cells produced a substantial amount of MFG-E8 compared to macrophages, ECs and melanoma cells. Furthermore, we confirmed that AS tumor cells implanted into mice expressed ECs marker CD31 and MFG-E8 by immunohistochemical staining. As MFG-E8 is secreted protein, MFG-E8 was also stained in interstitial area. These results suggest that AS tumor cells-derived MFG-E8 may play roles in the regulation of tumor growth in an autocrine and paracrine manner.

It had been reported that AS tumor cells expresses both VEGF and VEGF receptors, and AS is thought to be dependent on VEGF signaling for proliferation, invasion and metastasis [36,37]. However, Hoshina et al. reported that neither blocking of VEGF nor VEGF receptors lead to the inhibition of AS growth, suggesting that different angiogenic signaling might function in AS [32]. We found that depletion of MFG-E8 by siRNA did not suppress the production of VEGF, ET-1 and angiopoietin in AS cells. On the other hand, we previously examined the role of MFG-E8 in the production of angiogenic factors in MSCs *in vitro* using MFG-E8 KO mice-derived BM-MSCs, and identified that depletion of MFG-E8 decreased the production of ET-1 and VEGF in MSCs [18]. This discrepancy may be partially explained by the difference of cell types, however, further investigation is required.

In this study, we identified that cell proliferation of AS cells was not changed by MFG-E8 siRNA. This result is consistent with our previous result that cell proliferation of MFG-E8 KO MSCs was not changed compared to that of WT MSCs [18].

Regarding the mechanisms of the regulation of angiogenesis by MFG-E8, it has been reported that MFG-E8 acts on pericytes and ECs by potentiating the stimulatory effects of PDGF and VEGF, respectively, leading to enhanced angiogenesis [15,16,38]. We identified that depletion of MFG-E8 in AS cells by siRNA inhibited cell migration and capillary-like formation, suggesting that AS cells-derived MFG-E8 may enhance VEGF signaling in AS cells in an autocrine and paracrine manner, resulting in the enhancement of cell migration and capillary-like formation.

Combination therapy with systemic MFG-E8 blockade using an anti-MFG-E8 Ab and cytotoxic chemotherapy, molecular targeted therapy, or radiation inhibited the growth of murine tumors, including melanoma [14]. In the current study, we identified that anti-MFG-E8 Ab significantly inhibited the size of AS tumors in mice and the number of $CD163^+$ TAMs in AS tumors. It is generally recognized that infiltrating M2 type TAMs play an essential role in the progression of tumor growth, facilitating angiogenesis and the inhibition of antitumor immunity. With regard to TAMs and MFG-E8, MFG-E8 induced the efferocytosis of apoptotic prostate cancer cells and promoted M2 phenotype polarization in macrophages [28]. We previously demonstrated that the number of TAMs in melanoma tumors co-implanted with MFG-E8 KO-MSCs were significantly reduced compared with that in tumors co-implanted with WT-MSCs, suggesting that MFG-E8 might regulate the functions of TAMs [18]. Therefore, our results in this study suggest that MFG-E8 might enhance tumor growth of AS by increasing the number of TAMs in tumors.

The implanted AS tumors grew into the dark reddish tumors, and the size of the tumor peaked at 3–4 days after implantation, then it gradually shrunk and disappeared, suggesting that antitumor immunity, including $CD8^+$ T cells might be associated with the rejection of AS tumor in mice. Several studies showed the regulation of antitumor immunity by MFG-E8 [14,23,39]. Jinushi et al. reported that MFG-E8 inhibited the vaccination activity of GM-CSF-secreting tumor cells through regulatory T cells (Treg) induction, and that the combination anti-MFG-E8 Ab and chemotherapy enhanced antitumor effector T cells and inhibited Treg [14,39]. Kanemura et al. also suggests that high MFG-E8 expression in esophageal tumor might induce Treg propagation to suppress antitumor immunity exerted by $CD8^+$ T cells [23]. In our study, the number of $CD8^+$ T cells in AS tumors in mice treated with anti-MFG-E8 Ab tended to be increased, however, the difference was not significant. We also examined the number of $Foxp3^+$ Treg in AS tumor, however, we could not detect $Foxp3^+$ cells by immunohistochemical staining unfortunately.

Several studies have indicated that the expression of MFG-E8 is associated with the tumor growth and the survival of patients [22–30]. Consistent with the results of previous studies, we found that high MFG-E8 expression in human AS tumor was associated with the increased tumor growth and infiltration of TAMs, as well as poor prognosis in patients with AS. There has been reported several mechanisms that MFG-E8 promotes tumor growth, including the enhancement of angiogenesis [15,18,24], migration of tumor cells [25,27], proliferation of tumor cells [29], M2 macrophages polarization [18,28], as well as the suppression of anti-cancer immunity [23,26]. From our results, in AS tumors, MFG-E8 might promote tumor growth by the enhancement of angiogenesis, tumor cells migration and M2 macrophages polarization.

In conclusion, we, for the first time, identified the association of MFG-E8 and tumor growth of murine and human AS. AS-derived MFG-E8 might enhance tumor growth via angiogenesis and the induction of TAMs in autocrine/paracrine manner. These regulations by MFG-E8 might provide new insights into the pathogenesis of tumor growth in AS.

In addition, we identified the inhibition of tumor growth of AS in mice by anti-MFG-E8 Ab, suggesting that the blockade of MFG-E8 signaling could be a therapeutic potential for AS. However, additional studies regarding the role of MFG-E8 in human AS are warranted.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.08.005.

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