Interleukin-6 As An Enhancer of Anti-Angiogenic Therapy for Ovarian Clear Cell Carcinoma.

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Abstract

Ovarian clear cell carcinoma (OCCC) is a subtype of epithelial ovarian cancer (EOC) and associated with elevated interleukin-6 (IL-6) expression, resistance to chemotherapy, and increased mortality. Although bevacizumab (Bev) is a widely used anti-angiogenic agent for EOC, the efficacy of Bev and the role of IL-6 in modulating angiogenesis in OCCC are unknown. We performed tube formation assays on human umbilical vein endothelial cells (HUVEC) cultured in OCCC conditioned media and in direct co-culture with OCCC cells and observed that IL-6 inhibition significantly mitigated the ability of Bev to impede tube formation in both cases. Furthermore, the IL-6 blockade not only disrupted the anti-angiogenic efficacy of Bev but also the concomitant anti-tumor activity. We also found that IL-6 inhibition resulted in a significant increase in the secreted levels of angiopoietin-1 (Ang1) in addition to decreased vascular endothelial growth factor (VEGF) expression. Clinical specimens also exhibited this reciprocal relationship between IL-6 and Ang1 expression. Finally, knockdown of Ang1 prevented IL-6 inhibition from mitigating the effects of Bev, demonstrating that IL-6 supports the anti-angiogenic activity of Bev by suppressing Ang1 and promoting dependence on VEGF for angiogenesis. Altogether, our data suggest that OCCC tumors with high IL-6 are candidates for Bev therapy.

Introduction

Among the five distinct histotypes of epithelial ovarian cancer (EOC), ovarian clear cell carcinoma (OCCC) exhibits unique biological and molecular features, and therefore is recognized as a distinct entity presenting unique challenges for treatment ¹. The prevalence of OCCC varies by region; while accounting for only 1–12% of EOC cases in Western counties, OCCC is relatively frequent among Asian EOC patients ². Until recently, few clinical trials have examined OCCC specifically and there is insufficient evidence regarding optimal chemotherapeutic approach, including molecular medicine. In general, OCCC is less sensitive to platinum-based first-line chemotherapy than other EOC histotypes and is associated with poor prognosis in advanced cases ³. Identification of novel molecular targets related to carcinogenesis may help in patient stratification, prognosis, and treatment decisions. The AT-rich interactive domain 1A gene (*ARID1A*) and the phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit α gene (*PIK3CA*) are frequently mutated in OCCC, and co-mutation in transgenic mice promotes OCCC tumor initiation, possibly by continuous stimulation of interleukin-6 (IL-6)-dependent pro-inflammatory and pro-tumorigenic signaling as IL-6 is elevated in OCCC patients ⁴. Moreover, the potential involvement of elevated IL-6/signal transducer and activator of transcription 3 (STAT3) pathway activity in OCCC pathogenesis has been reported by several groups ^{5,6} and so may be a promising therapeutic target.

The anti-angiogenic drug bevacizumab (Bev), a monoclonal antibody against human vascular endothelial growth factor (VEGF), has been incorporated into first-line chemotherapy and follow-up maintenance therapy for advanced EOC. Although a randomized phase III trial evaluating the clinical benefits of Bev for EOC treatment (GOG-0218) did not show improvement in overall survival ⁷, a recent retrospective plasma sample biomarker analysis from GOG-0218 patients found that high serum IL-6

concentration predicted better anti-angiogenic treatment efficacy⁸. Thus, serum IL-6 may help to identify patients most responsive to anti-angiogenic treatment. Further, these findings suggest that IL-6 signaling may potentiate the anti-angiogenic actions of VEGF blockade by Bev.

In this study, we examined the modulatory actions of IL-6 on Bev anti-angiogenic efficacy by conducting tube formation assays in co-cultures of OCCC cell lines and human umbilical vein endothelial cells (HUVEC) in the presence or absence of Bev and/or anti-IL-6 antibody. Results suggested that IL-6 promotes the anti-angiogenic efficacy of Bev by suppressing angiopoietin-1 (Ang1) release. Further, we present evidence that IL-6 also suppresses Ang1 production in human OCCC tissue. Collectively, these results provide valuable information for the development of individualized OCCC treatment strategies.

Results

Attenuation of Bev anti-angiogenic efficacy by IL-6 signal blockade

To investigate the influence of IL-6 signaling on anti-angiogenic therapy, we first examined the capacity of HUVEC to form tubular networks in the presence of conditioned medium from the OCCC cell line RMG-1, which is known to secrete IL-6 (Fig. 1A, Supplementary Fig. 1S). Briefly, RMG-1 cells were cultured in 1% oxygen with or without anti-IL-6 antibody and the supernatant (conditioned medium) applied for tube formation assays. The ability of HUVEC cells to form tubular networks in RMG-1 conditioned media without IL-6 signal inhibition was equivalent to that in recombinant VEGF-supplemented culture medium as indicated by tube area measurements (Fig. 1A, B). As expected, addition of Bev strongly impeded tube formation both in VEGF-supplemented medium and RMG-1 conditioned medium without IL-6 signal inhibition, suggesting that RMG-1 cells may also secrete VEGF (Fig. 1B). However, the efficacy of Bev to impede tube formation was strongly mitigated by RMG-1 condition medium with anti-IL-6 antibody, from $62\% \pm 6.82\%$ in the absence of IL-6 signal blockade to only $24\% \pm 3.62\%$ in the presence of anti-IL-6 ($p = 0.001$) (Fig. 1C). Moreover, this mitigation by anti-IL-6 antibody was dose dependent (Supplementary Fig. 2S A, B).

Attenuation of Bev anti-angiogenic and anti-tumor efficacy under IL-6 signal blockade

These findings (Fig. 1) suggest that IL-6 signaling potentiates the anti-angiogenic efficacy of Bev. To clarify the underlying mechanisms as well as potential effects of IL-6 signaling on Bev anti-tumor activity, we examined these drugs in a co-culture system of the OCCC cell line RMG-1/GFP (emitting green fluorescence) and HUVEC pre-stained with DiI (emitting red fluorescence) (Fig. 2A). In this system, the tumor cell line attached to HUVEC and proliferated, implying a direct interaction (Supplementary Fig. 3S A). Consistent with the results shown in Fig. 1, Bev treatment alone for 4 days reduced tube area and this anti-angiogenic effect was significantly mitigated by anti-IL-6 ($p < 0.001$). Thus, reversal of Bev-mediated suppression of angiogenesis by IL-6 signal blockade was maintained in the presence of tumor cells.

Moreover, tumor area was also significantly larger in the anti-IL-6-treated group compared to controls ($p = 0.001$) (Fig. 2B), suggesting that IL-6 signal blockade not only disrupted the anti-angiogenic efficacy of Bev but also the concomitant anti-tumor activity. Both effects of IL-6 signal blockade on Bev activity were observed over the entire 10-day experimental period (Supplementary Fig. 3S B, C).

IL-6 signaling blockade enhanced Ang1 release from tumor cells

To further investigate the mechanisms underlying IL-6-dependent suppression of Bev anti-angiogenic activity, we examined the effects of IL-6 on secretion of the angiogenic modulators VEGF, Ang1/2, and osteopontin by tumor and HUVEC. Production of VEGF by RMG-1 cells was only mildly suppressed by anti-IL-6 antibody treatment as evidenced by ELISA analysis of culture supernatant (Fig. 3A), while osteopontin production by RMG-1 tumor cells was significantly suppressed by anti-IL-6 ($p < 0.01$) (Fig. 3B). In contrast, anti-IL-6 antibody treatment enhanced Ang1 production ($p < 0.05$) (Fig. 3C). HUVECs did not produce detectable VEGF or osteopontin, while Ang1 production was increased and Ang-2 production decreased by anti-IL-6 antibody treatment (Fig. 3A-D). These results suggest that the anti-angiogenic activity of Bev is mitigated by IL-6 signal blockade through enhanced production of Ang1 (rather than VEGF production). To further assess IL-6 effects on Ang1 production by OCCC cells, these ELISA assays were repeated on several other OCCC cell lines. In general, these assays indicated that IL-6 and Ang1 production are reciprocally regulated. While OVTOKO and HAC-2 lines produced little IL-6, both produced high levels of Ang1. Conversely, the OVISE line produced a large amount of IL-6 and a small amount of Ang1 (Supplementary Fig. 1S A). Only RMG-1 and -2 cells produced moderate amounts of both IL-6 and Ang1.

We then examined the relationship between IL-6 and Ang1 in 60 OCCC clinical tissue samples. Of 13 tumor samples with high IL-6 expression, only 1 sample also expressed a high level of Ang1 as evidenced by immunohistochemical staining. Further, Ang1 expression was significantly lower among tumor samples with high IL-6 expression compared to samples with low IL-6 expression ($p = 0.021$) (Supplementary Fig. 4S A, B). This result suggests that Bev may be more efficacious on tumors expressing high levels of IL-6 due to a suppressive effect of IL-6 on pro-angiogenic Ang1 release.

Suppression of Ang1 release by a targeted siRNA restored Bev anti-angiogenic efficacy

To confirm that IL-6 potentiates the anti-angiogenic efficacy of Bev by suppressing Ang1 release, we conducted tube formation assays under conditions of Ang1 manipulation using targeted and control siRNAs. First, we confirmed the feasibility of this strategy by demonstrating that the targeted siRNA (siAng1) indeed reversed Ang1 upregulation by IL-6 signal blockade (Supplementary Fig. 5S). In the conditioned medium from RMG-1 cells transfected with siAng1 and receiving anti-IL-6 treatment, Bev reduced the tube area by 55%, similar to assays conducted in conditioned medium from tumor cells receiving no anti-IL-6 treatment (Fig. 4A, B, C). In other words, suppression of Ang1 accumulation in the conditioned medium restored the anti-angiogenic efficacy of Bev in the presence of anti-IL-6, providing

further evidence that IL-6 promotes the anti-angiogenic activity of Bev indirectly by suppressing Ang1 secretion from OCCC cells.

Discussion

Molecular profiling of OCCC tumor tissues has revealed several potential prognostic biomarkers, predictors of treatment response, and therapeutic targets⁹⁻¹⁴. For instance, studies have reported the hyper-activation of several signaling pathways including hypoxia-inducible factor 1 α (HIF-1 α)/VEGF and IL-6/STAT3 pathways in OCCC¹⁵⁻¹⁷. Mabuchi et al. reported that OCCC cells under intratumoral hypoxia strongly expressed VEGF and that Bev demonstrated anti-tumor efficacy against OCCC both in vitro and in vivo¹⁸. However, they also suggested that VEGF may not be a reliable biomarker for predicting Bev sensitivity. Therefore, although anti-angiogenic treatment by Bev appears promising based on pathogenesis, there is still no widely recognized biomarker predictive of its clinical efficacy. Here we demonstrate that high IL-6 or low Ang1 may be such predictors.

Upregulation of IL-6 and related pathway mediators as well as the anti-tumor efficacy of IL-6 pathway inhibition have been reported in OCCC^{5,6,19,20}. In addition, several studies have documented significant associations between poor OCCC prognosis and high IL-6 in tumor or serum samples^{5,20,21}. Anglesio et al. reported upregulation of the IL-6/STAT3/HIF pathway and therapeutic responses to the anti-angiogenic agent sunitinib in two chemotherapy-resistant OCCC cases⁵. However, the phase II GOG-254 trial evaluating this multi-receptor tyrosine kinase inhibitor for the treatment of persistent or recurrent OCCC found minimal clinical efficacy²². Recently, a retrospective biomarker analysis revealed longer survival by Bev-treated patients with high plasma IL-6 level compared to those with lower plasma IL-6⁸. Based on these findings, we evaluated the influence of IL-6 on Bev anti-angiogenic and anti-tumor efficacies using an in vitro OCCC model to reveal potential molecular mechanisms, and further examined the association of IL-6 expression with that of the pro-angiogenic factor Ang1 among OCCC patients to help identify good candidates for Bev treatment.

In general, IL-6 has been shown to influence angiogenesis as pro-angiogenic factor²³. For instance, transgenic mice engineered to overexpress IL-6 exhibited hypervascularization of the cerebellum²⁴, while mice deficient in IL-6 exhibited reduced angiogenic responses to wound injury²⁵. Further, IL-6 induced VEGF production by tumor cells and ensuing angiogenesis^{26,27}. In the current study, IL-6 signal blockade reduced the anti-angiogenic activity and associated anti-tumor activity of Bev in vitro co-culture system with OCCC tumors and HUVEC. However, the moderate alteration of VEGF production by OCCC cells under IL-6 blockade led us to speculate that some other angiogenic factor(s) were responsible for the observed attenuation of Bev anti-angiogenic activity. Several VEGF-independent angiogenic mechanisms of IL-6 have been reported, including Ang1 modulation, and Kayakabe et al. reported that IL-6 could destabilize angiogenesis through inhibition of Ang1 signaling in a co-culture model of rheumatoid arthritis²⁸. Additional analyses in our model suggested that IL-6 promoted the anti-angiogenic activity of Bev by suppressing Ang1 release from OCCC cells. Furthermore, the association of high IL-6 expression with low

Ang1 expression was found in a series of OCCC patient specimens. According to these results, Ang1 may interfere with Bev function in OCCC (Fig. 5).

Angiopoietin-1 is thought to facilitate vessel stabilization and its signaling is thought to support the induction and growth of tumor vasculature even under VEGF blockade, resulting in improved tumor perfusion²⁹. Huang et al. also reported that Ang1 protected the tumor vasculature from regression, increased vessel caliber, and induced the recruitment of mural cells under anti-VEGF treatment³⁰. Moreover, Casanovas et al. reported that tumors showing continued progression during anti-VEGF receptor-2 antibody treatment maintained substantial Ang1 expression³¹. According to these evidence, Ang1 suppression may be important for Bev to exert its efficacy. In the current study, we found that IL-6 blockade enhanced Ang1 production by tumor cells, while siRNA-induced Ang1 knockdown restored the impaired anti-angiogenic function of Bev under IL-6 blockade. Collectively, IL-6 signaling could reduce the tumor angiogenic function of Ang1 and relatively increases that of VEGF, which in turn enhances Bev function in OCCC cells.

The utility of IL-6 as a potential predictor of anti-angiogenic drug response is still debated. Earlier reports suggested that high IL-6 levels could be predictive of survival benefit from Bev treatment in patients with EOC⁸, metastatic renal cancer³², and metastatic colorectal cancer¹⁰. In contrast, however, others reported that low IL-6 levels were associated with better Bev treatment response against hepatocellular carcinoma³³, pancreatic cancer³⁴, and metastatic colorectal cancer³⁵. In addition, a recent clinical study reported that aflibercept, a recombinant fusion protein that blocks the VEGF pathway in advanced EOC, was more effective in patients with low IL-6 levels³⁶. Differences in cellular IL-6 response among tumor types, study design, or clinical stage may explain these discordant results. Further research efforts are warranted to elucidate how IL-6 modulates Ang1 and the prognostic utility of IL-6 as a biomarker for anti-angiogenic drug response.

In conclusion, this study demonstrated that IL-6 enhanced the anti-angiogenic efficacy of Bev by suppressing Ang1 in addition to increased VEGF production in OCCC cells. Furthermore, the current study provides a strong rationale for prospective clinical trials of anti-angiogenic therapy for EOC including OCCC considering about IL-6 as well as Ang1 measurements to evaluate its prognostic utility.

Materials And Methods

Cell culture

The human OCCC cell lines RMG-1 and RMG-2 were kindly provided by Dr. D. Aoki (Keio University, Tokyo, Japan) and maintained in Ham's F12 (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS)(Cytiva, Marlborough, MA, USA). The OVTOKO and OVISE lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and the HAC-2 line was kindly provided by Dr. M. Nishida (Tsukuba University, Tsukuba, Japan). These three cell lines were maintained in RPMI-1640 medium (Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% FBS. For

experiments, all OCCC cell lines were pre-incubated for 24 h in M199 medium (Sigma–Aldrich) supplemented with 0.5% bovine serum albumin (BSA) (Sigma–Aldrich) under a hypoxic environment (1% O₂) created by the BIONIX hypoxic culture kit (SUGIYAMA-GEN, Tokyo, Japan). To modulate IL-6 signaling, the indicated concentration of anti-IL-6 antibody (MAB206-100, clone 6708; R&D Systems, Minneapolis, MN) was added to the medium and culture continued for 48 hours. Other cultures were incubated for the same period but without anti-IL-6 treatment. Subsequently, supernatants were collected as RMG-1 conditioned media (with or without anti-IL-6) and stored frozen for further experiments. An RMG-1 line expressing green fluorescent protein (RMG-1/GFP) was purchased from Anticancer Japan (Chiba, Japan) and treated in the same manner as RMG-1 cells. HUVEC was kindly provided by Dr. I. Yamamoto (The Jikei University School of Medicine, Tokyo, Japan) and maintained in endothelial cell medium (Sciencell Research Laboratory, Carlsbad, CA) with 5% FBS.

Transfection and siRNA

A predesigned small interfering RNA (siRNA) targeting angiopoietin-1 (siAng1) (Silencer™ Select Pre-Designed siRNA, Assay ID: s1356) and a non-targeted control siRNA (siCTL) (Silencer™ Select Negative Control, #4390843) were purchased from ThermoFisher Scientific (Waltham, MA). Prior to transfection, siRNAs were premixed with lipofectamine RNAiMAX (ThermoFisher Scientific) plus Opti-MEM (ThermoFisher Scientific). RMG-1 cells were then transfected by incubation with this mixture for 24 hours under a hypoxic environment. After an additional 24 hours of incubation, the culture medium was exchanged for fresh medium with or without 100 ng/mL anti-IL-6 antibody as indicated. The supernatants from these cultures were collected as conditioned media for tube formation assays.

Antibodies and angiogenic factor

Recombinant human VEGF (aa207-318) was purchased from PeproTech (Rocky Hill, NJ). The human VEGF-A monoclonal antibody Bev was kindly provided by Chugai Pharmaceutical Co., Ltd (Tokyo, Japan).

Tube formation assay

Tube formation assays were conducted as described by Yin et al.³⁷. Briefly, Corning 96-well flat bottom plates (Corning, NY) were coated with 30 µL growth factor-reduced Matrigel matrix (Corning) and seeded with 1×10^5 HUVEC in 100 µL RMG-1 conditioned medium supplemented with 0.5% BSA. Recombinant VEGF (5 ng/mL) and Bev (10 µg/mL) were added as indicated for specific experiments. After 18 hours of incubation, tubes were stained with Calcein AM (Corning) to quantify total tube area using a fluorescence microscope (BZ-X800, Keyence, Osaka, JAPAN) and the BZ-H4C analytic application for hybrid cell count and the BZ-H4CM application for macro cell count.

Enzyme-linked immunosorbent assays (ELISAs)

Enzyme-linked immunosorbent assays (ELISAs) were performed to measure the medium concentrations of angiogenic factors secreted by RMG-1, RMG-2, OVISE, OVTOKO, and HAC-2 cell lines. Briefly, cells were seeded in T-25 flasks at 5×10^5 cells per flask. After 24 hours of incubation, the culture medium was exchanged for M199 medium plus 0.5% BSA with or without anti-IL-6 antibody (100 ng/mL) as indicated

and incubation continued under hypoxia for an additional 48 hours. Part of the culture supernatant was then collected for ELISA analysis of VEGF, IL-6, Ang1, angiopoietin-2 (Ang2), and osteopontin concentrations using specific ELISA kits (DVE00 for VEGF, DANG10 for Ang1, DANG20 for Ang2, and DOST00 for osteopontin; all from R&D Systems). The remaining supernatants were collected as conditioned media for HUVEC culture. HUVEC was initially seeded at 1×10^5 cells per T-25 flask. After 24 hours of incubation, the culture medium was exchanged for RMG-1 cell conditioned medium and incubation continued for an additional 48 hours. The supernatants were then collected for analysis using the same ELISA kits.

Co-culture assay

A 3D-culture protocol was performed based on the Matrigel sandwich structure method described previously³⁸. Briefly, 30 μ L Matrigel was added into each well of a 96-well plate pre-chilled on ice. The plates were then incubated at 37 °C for 30 min to enable polymerization of this Matrigel basal layer. HUVEC cells were pre-stained with Corning® DilC12(3) Fluorescent Dye (Dil), suspended at 2×10^4 cells per 75 μ L M199 medium supplemented with 1% FBS, and seeded onto the basal layer. After 4 hours of incubation to allow tube formation, the same number of RMG-1/GFP cells suspended in 75 μ L pre-chilled M199 medium containing 1% FBS, 10 μ g/mL Bev, and 10% Matrigel with or without 100 ng/mL anti-IL-6 antibody were seeded onto the polymerized Matrigel base layer. Then, the plates were incubated at 37 °C to allow polymerization of the top layer. The co-culture system was maintained in M199 medium containing 1% FBS at 37 °C under a 5% CO₂ atmosphere for 10 days with medium exchange every other day. The Dil-stained area and GFP-stained area, corresponding to tube area and tumor area, respectively, were quantified using a fluorescence microscope (BZ-X800, Keyence) and the BZ-H4C and BZ-H4CM analytic applications.

Clinical samples and immunohistochemistry

Analysis of human tumor samples was approved by the ethics committee of The Jikei University School of Medicine (32 – 017(10092)). Sixty stage II–IV OCCC patients receiving primary surgical resection followed by post-operative adjuvant chemotherapy at The Jikei University School of Medicine and affiliated hospitals from 2013 to 2018 were enrolled. Paraffin-embedded tumor samples from primary surgery were stained with hematoxylin-eosin to confirm the diagnosis of OCCC. To examine IL-6 and Ang1 expression, formalin-fixed, paraffin-embedded tissue sections (4 μ m thick) were deparaffinized, incubated in Cell Conditioning 1 (CC1) standard solution (citrate buffer pH 8.5, Ventana Medical Systems) for 60 min at 100 °C for antigen retrieval, and then incubated in antibodies against IL-6 (1:400, 21865-1-AP; Proteintech) and Ang1 (1:200, ab8451; Abcam, Cambridge, UK). Immunostained slides were evaluated by two independent pathologists (H.M, M.M) blinded to clinical information. Staining scores were standardized by comparison between the observers, and discrepancies were resolved by reevaluating the slides using a multi-head microscope. Immunoreactivity for IL-6 in the tumor cytoplasm was categorized as none/focal (0–40%) or diffuse (50–100%)¹¹. Immunostaining for Ang1 was assessed in a semi-quantitative manner as previously described³⁹. Tumor and stromal areas were

evaluated independently and each section was assigned two scores: Staining intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, intense staining) and proportion of cells stained (0, no cells staining; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%). The scores for staining intensity and proportion of stained cells were multiplied to yield individual tumor and stromal area staining scores for Ang1. The two scores were then added to yield a final Ang1 staining score for each sample, which was then categorized as high (> 8) or low (0–7).

Statistical analysis

All data are expressed as the mean $\pm$ standard error of the mean. Means of experimental and control groups were compared by independent samples Student's t-test, one-way analysis of variance followed by post hoc Bonferroni's multiple comparison tests, or Fischer's exact tests as indicated. A $p < 0.05$ (two-tailed) was considered statistically significant for all tests. All statistical analyses were performed using EZR software (Saitama Medical Centre, Jichi Medical University; <http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedOSX.html>, Kanda, 2012), a graphical interface for R (The R foundation for Statistical Computing. Vienna, Austria, ver. 3.2.2).

Declarations

Authors' contributions

Conception and design: N.Y, T.S, J.S.S

Acquisition of data: T.S, M.S, D.N, H.M, M.M, A.K, M.T

Analysis and interpretation of data: N.Y, M.S, T.S

Writing, review, and/or revision of the manuscript: T.S, N.Y, J.T, R.Y, T.K, J.S, K.T, Y.I, S.Y, J.S.S, A.O

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Conflict of Interest statement:

The authors declare no potential conflicts of interest.

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Figures

A **B** **C** **Figure 1**

Attenuation of anti-angiogenic function of Bev in culture media of IL-6 blocked OCCC cell. (A) Representative images of tube formation of HUVEC with the indicated culture media and reagents (magnification x 100). Fluorescent microscope observation was made after 18 hours of incubation and Calcein AM staining. (B) Tube area in each well with indicated condition were measured by hybrid cell count software. Data were average of triplicated well. (C) The tube reduction rate by Bev treatment with or without IL-6 blockade. IL-6 blockade weakened Bev function. Data are shown from one of two independent experiments with similar results. Error bars are SEs.

A **B** **Figure 2**

The role of IL-6 signal for the anti-angiogenic and anti-tumor effects of Bev in 3D co-culture system. (A) Representative micrograph of 3D co-culture with or without IL-6 blockade observed through light microscope and fluorescent microscope at experimental day 4 after Bev treatment. HUVEC was stained by Dil, emitting red color. RMG-1/GFP was green color. Overlaid images of Dil and GFP were shown at the rightmost. (B) Measured area at day 4 of each color by hybrid cell count software. Dil stained area, regarded as tube area, is shown on the left and GFP area, regarded as tumor area, is shown on the right side. Error bars are SEs.

Figure 3

Angiogenic factors released by RMG-1 and HUVEC. (A) VEGF, (B) osteopontin, (C) Ang1, and (D) Ang2 production from RMG-1 and HUVEC in response to IL-6 signal blockade determined by ELISA assay. Data of HUVEC was calculated by the subtraction the data of RMG-1 mono-culture from that of RMG-1+HUVEC. Error bars are SEs. *P<0.05, **P<0.005.

Figure 4

The role of Ang1 in IL-6 mediated Bev anti-angiogenic effect enhancement. (A) Images of tubes formed by HUVEC in the conditioned media from RMG-1 treated by indicated siRNA and anti-IL-6 antibody (magnification x 40). Fluorescent microscope observation was made after 18 hours of incubation and Calcein AM staining. (B) Tube in each well with indicated condition were measured by hybrid cell count software in order to figure out tube area. Data were average of triplicated well. (C) Reduction rate of tube area by Bev treatment with or without IL-6 blockade and siRNA, IL-6 blockade weakened Bev function in siCTL whereas siAng1 restored the anti-angiogenic function. Data are shown from one of two

independent experiments with similar results. siCTL: siRNA without gene silencing ability (control).
siAng1: angiopoietin-1 silencing siRNA. Error bars are SEs.

Figure 5

Mechanism of enhanced anti-angiogenic efficacy of Bev in IL-6 high tumor with Ang1 suppression.

Supplementary Files

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