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Interaction of Mesenchymal Stem Cells with Fibroblast-like Synoviocytes via Cadherin-11 Promotes Angiogenesis by Enhanced Secretion of Placental Growth Factor

Su-Jung Park,* Ki-Jo Kim,† Wan-Uk Kim,*† and Chul-Soo Cho*‡

Bone marrow–derived mesenchymal stem cells (MSC) exist in the synovium of patients with rheumatoid arthritis (RA), yet the role of MSC in RA is elusive. Placental growth factor (PIGF) expression is increased in RA synovial fluids, and blocking of PIGF attenuates progression of arthritis in mice. In this study, we observed that PIGF induced chemotaxis of MSC in a dose-dependent manner, which was blocked by anti–vascular endothelial growth factor receptor-1 peptide. MSC exposed to PIGF elicited increased phosphorylation of Akt and p38 MAPK. PIGF-mediated chemotaxis was inhibited by PI3K inhibitor (LY294002) and p38 MAPK inhibitor (SB203580), but not by ERK1/2 inhibitor (PD98059). Fibroblast-like synoviocytes (FLS) constitutively produced PIGF, but MSC released negligible amounts of PIGF. Of note, when FLS of RA patients and MSC were cocultured, PIGF production by FLS was significantly increased; such an increase was dependent on the number of added MSC. Moreover, coculture conditioned medium promoted chemotaxis of MSC and increased angiogenesis in Matrigel plugs assay, and these were suppressed by preincubation of the medium with anti-PIGF Ab. Transwell experiments revealed that MSC to FLS contact was required for the increase in PIGF production by coculture. Cadherin-11 was expressed both in FLS and MSC, and small interfering RNA knockdown of cadherin-11 in FLS significantly abrogated the enhanced PlGF production under coculture conditions. These data indicate that increased levels of PIGF in RA joints could induce the migration of MSC to the synovium, and interaction of migrated MSC with FLS via cadherin-11 may contribute to angiogenesis and chronic synovitis by enhancing the secretion of PIGF. The Journal of Immunology, 2014, 192: 3003–3010.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of synovial lining cells, infiltration of mononuclear cells, and abundant new vessel formation in the synovium (1). Angiogenesis has been considered to be a critical step in the initiation and perpetuation of synovitis, and a variety of angiogenic factors involved in RA pathology has been identified to date (2, 3). Placental growth factor (PIGF) is a 25-kDa dimeric protein that is highly homologous with vascular endothelial growth factor (VEGF) (4) and is detected at higher levels in RA synovial fluid (SF) than in osteoarthritis (OA) SF (5). PIGF is not only mitogenic for endothelial cells (ECs) (6) but also induces inflammatory responses by increasing the production of TNF-α, IL-1, IL-8, and MCP-1 by cultured monocytes (7). We previously demonstrated that blocking PIGF either by a novel anti–VEGF receptor-1 (VEGFR-1) hexapeptide, GNQWFI, or genetic deletion inhibited arthritis progression in mice, indicating the pivotal role of PIGF in the pathogenesis of RA (5).

Mesenchymal stem cells (MSC) are nonhematopoietic stromal cells that can differentiate into bone, cartilage, muscle, ligament, tendon, and adipose tissue (8). MSC are regarded as a promising cell type for regenerative medicine because they show high migratory capacity toward inflamed or remodeling tissues through a number of adhesion molecules and chemokine receptors (9). In addition, they are explored as a therapeutic option for treating a variety of immune diseases by their immunosuppressive features (10). However, the detrimental role of MSC has been also reported. For example, MSC have been known to migrate toward primary tumors and metastatic sites and contribute to the progression of tumors by affecting tumor cell survival and angiogenesis (11).

Bone marrow (BM)–derived MSC exist in the synovium of arthritic joints (12, 13), yet the precise role of MSC in RA pathology remains elusive. In experimental arthritis, joint inflammation is preceded by infiltration of MSC, which may contribute to the hyperplasia of synovial cells (14). In addition, the arthritic score in mice with collagen-induced arthritis is further increased by intra-articular injection of MSC (15). Moreover, arthritic and aggressive synoviocytes contain a substantial (>30%) fraction of BM-derived precursors (16), which suggests that recruitment of MSC into the joints is crucial to the development of synovial hyperplasia in mice with chronic arthritis. However, it remains to be determined what factors are responsible for MSC migration to the joints. Given the high concentration of PIGF in RA joints and its promigratory potential, MSC might be recruited into arthritic joints by the effect of PIGF, interact with resident RA synoviocytes via cell-to-cell contact or by the secretion of a variety of cytokines and angiogenic factors, including PIGF, and thereby...
promote RA inflammation and angiogenesis. To test such possibilities, we investigated if PIGF secreted by RA synoviocytes could increase MSC migration and tested the effect of interaction of MSC with RA synovial fibroblasts on PIGF production and angiogenesis.

Materials and Methods
Isolation of human BM-derived MSC
After informed consent, BM-derived MSC were prepared from leftover material obtained from normal individuals (n = 10) undergoing marrow harvests for allogeneic transplantation, as approved for this study by the institutional review board of Yeouido St. Mary’s Hospital (SC12TISI0061). Mononuclear cells were isolated by Ficoll density-gradient centrifugation at 2500 rpm for 30 min, washed (twice with PBS, and seeded at 2 × 10^5 cells in T175 tissue culture flasks (BD Biosciences)). After 1 wk of culture in low-glucose DMEM supplemented with 10% FCS (Life Technologies), nonadherent cells were removed, and the medium was replaced every 3 d until the cells were confluent. These cells were then passaged up to three times, and MSC at passages 3–5 were used in our experiments after characterization of MSC by flow cytometric analysis (17). The following Abs used in this study were obtained from BD Biosciences: PE-conjugated anti-CD34 (clone 5G10, 553796; 1:20 dilution), PE-conjugated anti-CD73 (clone AD2, 550257; 1:50 dilution), and FITC-conjugated anti-HLA-DR (clone G46-6, 558111; 1:50 dilution). The following were purchased from eBioscience: allophycocyanin-conjugated CD34 (clone 4H11, 17-0349; 1:20 dilution), PerCP-Cy5.5-conjugated anti-CD45 (clone H300, 45-0045; 1:20 dilution), PE-conjugated anti-CD29 (clone TS2/16, 12-0299; 1:25 dilution), PE-conjugated CD14 (clone 6D3, 8012-0149-025; 1:25 dilution), and phycoerythrin-conjugated CD105 (clone SN6, 17-1057; 1:20 dilution).

Analysis of VEGFR-1 expression and its phosphorylation
Western blotting and RT-PCR analysis for expression of VEGFR-1 were performed as described previously (18). Surface expression of VEGFR-1 on MSC was assessed by flow cytometry. MSC (1 × 10^6) were stained with the PE-labeled mouse anti-human VEGFR-1 Ab (clone 49560, 1:25 dilution) for 30 min at 4˚C in the dark. Non-specific binding was blocked with PE-labeled mouse anti-human I gG1 FAB321P; R&D Systems) at 1:25 dilution for 30 min at 4˚C in the dark.

Chemotaxis assay of MSC
Chemotaxis assays were conducted in 48-well chemotaxis chambers (Neuroprobe). The contents of the upper and lower chambers were separated by polycarbonate filters (8-µm pore size), MSC (3 × 10^5) derived from healthy donors were resuspended in DMEM supplemented with 1% FCS (DMEM/1% FCS) and seeded in the upper wells. In selected wells, MSC were pretreated separately with 10 ng/ml recombinant PIGF (R&D Systems), and cell lysates were immunoprecipitated with rabbit anti-human VEGFR-1 Ab (clone Y103, ab32152, Abcam) and control rabbit IgG. The immunoprecipitated protein was immunoblotted with mouse anti-phosphotyrosine Ab (clone PY99, sc7020; Santa Cruz Biotechnology) and total/ phospho-ERK1/2 were purchased from Cell Signaling Technology. The density of the blots was scanned and quantified using ImageJ software (National Institutes of Health).

Isolation and culture of fibroblast-like synoviocytes
The fibroblast-like synoviocytes (FLS) were prepared from the synovial tissues of patients with RA who had undergone total joint replacement surgery. The study protocol was approved by the institutional review board of Yeouido St. Mary’s Hospital (SC12TISI0061), and informed consent was obtained from each patient for research uses of the tissue. The isolation of FLS from the synovial tissues was performed according to a procedure described previously (20). FLS, from passages 3–6, were used for each experiment. The purity of FLS was examined by flow cytometric analysis; these cells were <1% CD14 (clone 61D3), <1% CD3 (clone OKT3), <1% CD19 (clone HIB19; all from Biorad), and >98% CD90 (clone 5E10; BD Bioscience).

Coculture of BM-derived MSC and FLS
Coculture of MSC on a monolayer of FLS was performed in 24-well plates. FLS (1.5 × 10^5) were seeded in DMEM supplemented with 10% FCS and allowed to adhere overnight. Cells were then washed with serum-free DMEM, and suspensions of MSC (ranging from 1.5 × 10^4 to 1.5 × 10^5) were added either directly onto the FLS or into the upper chamber of a Transwell apparatus (Costar), which physically separates the MSC from the FLS, but allowed for interaction between the cells via soluable factors. Each cell population was also cultured alone. For blocking experiments, selected cocultures were treated with neutralizing mouse mAbs (20 µg/ml) to V CAM-1 (clone BBIG-V1, BBA5; R&D Systems) and ICAM-1 (clone BBIG-11, BBA3; R&D Systems) for different time periods, the supernatants were harvested and centrifuged to remove cellular debris. The cell-free culture supernatants were assayed for PIGF by a commercial ELISA kit (R&D Systems).

In vivo Matrigel plug assay
To prepare the conditioned medium (CM), FLS of RA patients (RA-FLS, 2 × 10^5) were plated in 24-well plates containing DMEM medium supplemented with 10% FCS and grown overnight. Cells were then washed with PBS, and an equal number of MSC were then added to plates containing DMEM supplemented with 1% FBS at a volume of 300 µl. After 48 h of coculture, CM were harvested, centrifuged at 1000 × g for 5 min at 4˚C, and filtered through a 0.20-mm pore syringe filter to remove cell debris.

Matrigel plugs were assayed as described previously (21). Briefly, C3HBL/6 mice were inoculated with 1 × 10^5 Matrigel (BD Biosciences) containing 30 U/ml heparin and 100 µM CM either from FLS culture or FLS-MSC coculture. For the blocking experiment, coculture CM was preincubated for 1 h with neutralizing mouse mAb (70 µg/ml) to PIGF (clone 37203, MAB264; R&D Systems) before mixing with Matrigel. After 14 d, the mice were sacrificed, and the Matrigel plugs were removed and analyzed for vascularity. Hemoglobin contents were measured. After cooling a Drakbin reagent kit (Sigma-Aldrich) and were expressed as micrograms per milliliter of hemoglobin per gram of Matrigel. Some of the Matrigel plugs were fixed in 4% formalin, embedded with paraffin, and stained using H&E.

Western blot analysis
Rabbit polyclonal Abs against total/phospho-Akt, total/phospho-p38, and total/phospho-ERK1/2 were purchased from Cell Signaling Technology. Mouse mAb against cadherin-11 (clone 16A; ab78477) was purchased from Abcam. Cellular proteins from MSC and FLS under various treatments were resolved by 8–12% SDS-PAGE and probed with different primary Abs as specified above. HRP-conjugated secondary Abs (anti-mouse or anti-rabbit) were used in conjunction with an ECL chemiluminescence detection system (Amersham). Loading of equal amounts of proteins on gels was confirmed by reprobing the membranes with β-actin and corresponding nonphosphorylated Abs. The density of the blots was scanned and quantified using ImageJ software (National Institutes of Health).

Small interfering RNA transfection
Cadmherin-11 small interfering RNA (siRNA) and control siRNA were purchased from Santa Cruz Biotechnology. Briefly, FLS were seeded in 24-well plates at 1.5 × 10^5 cells/well in DMEM supplemented with 10% FBS and grown to 50–70% confluence. Cells were then transfected with siRNA by Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After overnight stabilization, transfected cells were treated with or without TGF-β and then cocultured with MSC.

Statistical analysis
All results are expressed as means ± SD. Comparisons of numerical data between groups were performed by t test. The p values <0.05 were considered significant.
**Results**

**Expression of VEGFR-1 in BM–derived MSC**

Human BM-derived MSC were first investigated for the presence of MSC-related cell surface Ags by flow cytometric analysis. As seen in Fig. 1A, the cells were positive for CD90, CD29, CD73, and CD105, but negative for the hematopoietic lineage markers CD34, CD45, CD14, and HLA-DR (Fig. 1A). BM-derived MSC from four different healthy subjects constitutively expressed the mRNA encoding VEGFR-1, but not VEGFR-2, as determined by RT-PCR. The protein expression of VEGFR-1 was confirmed by Western blotting and flow cytometric analysis (Fig. 1B). Next, to assess whether PlGF induces receptor phosphorylation, lysates of MSC stimulated with recombinant PlGF (10 ng/ml) were immunoprecipitated with anti-VEGFR-1 Ab and immunoblotted with phosphotyrosine-specific mAb. The result showed that PlGF treatment increased VEGFR-1 phosphorylation in MSC (Fig. 1C), which peaked at 30 min and decreased at 60 min (data not shown). Additionally, PlGF-induced phosphorylation was blocked by anti–VEGFR-1 hexapeptide, which is known to specifically block the interaction of VEGF or PlGF with VEGFR-1 by selectively binding to VEGFR-1 (22).

**Effect of PlGF on the chemotaxis of MSC**

Because PlGF binds to VEGFR-1, a receptor involved in migration of ECs (23), we tested if PlGF promotes MSC migration. As shown in Fig. 2A, recombinant PlGF induced MSC chemotaxis in a dose-dependent manner, as determined by chemotaxis assay. Moreover, treatment with anti–VEGFR-1 hexapeptide inhibited PlGF-induced MSC chemotaxis in a dose-dependent manner with an approximate IC50 value of 20 μg/ml (p < 0.005), indicating the requirement of VEGFR-1 for PlGF-induced chemotaxis of MSC. We next investigated the downstream signaling pathways leading to MSC chemotaxis. In human monocytes, PlGF binding to VEGFR-1 triggers multiple intracellular signaling pathways, including PI3K, Akt, ERK1/2, and p38 MAPK, and these signal transducers are implicated in the regulation of chemotaxis (24). To this end, MSC were stimulated with PlGF in the presence of pharmacologic inhibitors specific for p38 MAPK, ERK1/2, and PI3K, and a chemotaxis assay was then performed. As a result, PlGF-induced chemotaxis was significantly decreased by p38 MAPK inhibitor SB203580 (2–10 μM) and PI3K inhibitor LY294002 (5 μM). However, neither ERK1/2 inhibitor PD98059 (1–50 μM) nor JNK inhibitor SP600125 (1–25 μM) affected MSC chemotaxis in response to PlGF (Fig. 2B). These agents were not toxic at the concentrations used for the experiments, as indicated by Cell Counting Kit-8 assay (Dojindo Molecular Technologies).

Consistent with these data, recombinant PlGF triggered phosphorylation of p38 MAPK and Akt, but had little influence on the level of phospho-ERK1/2 (Fig. 2C) and phospho-JNK1/2 (data not shown). In addition, PlGF-triggered phosphorylation of p38 MAPK was abrogated by p38 inhibitor SB203580, but not by PI3K inhibitors LY294002 and wortmannin (10 μM). In contrast, Akt phosphorylation following PlGF treatment was nearly completely blocked by PI3K inhibitors, which is in parallel with earlier reports that PI3K signaling precedes Akt activation (24). In addition, PlGF-triggered phosphorylation of Akt was also abrogated in the presence of SB203580, suggesting that Akt is a downstream effector of p38 MAPK. Collectively, our data indicate that PlGF induces VEGFR-1–mediated migration of MSC, which is dependent on the activation of both the PI3K/Akt and p38 MAPK signal pathways.

**Enhanced secretion of PlGF by coculture of MSC and FLS**

As we reported previously (5), RA-FLS constitutively expressed significant levels of PlGF (Fig. 3A). In contrast, BM-derived MSC produced negligible amounts of PlGF (Fig. 3A). Of note, coculture of RA-FLS with MSC at an equal density ratio induced a synergistic increase in the secretion of PlGF compared with the sum of its secretion from the two cell types cultured separately (Fig. 3A). The effect was evident at 24 h and persisted up to 72 h (maximum periods of coculture). When FLS were cocultured with a different number of MSC (MSC/FLS ratio ranged from 0.1–1) for 48 h, a graded increase in PlGF secretion was detected in the culture medium (Fig. 3B).
In RA joints, resident FLS are exposed to several inflammatory mediators, some of which have potent angiogenic activity (25, 26). We thus investigated the effect of inflammatory cytokines found at high level in the RA joints on the production of PlGF. Addition of TNF-α and TGF-β on either FLS or MSC failed to increase the production of PlGF in these cells (data not shown). However, the addition of TGF-β during coculture further increased the production of PlGF compared with the TGF-β–untreated control (Fig. 3C). The addition of either IL-1β or TNF-α during coculture also increased the production of PlGF, but the effects were less potent compared with TGF-β treatment (Fig. 3C). To determine whether direct contact of MSC to FLS is necessary for enhanced secretion of PlGF, MSC were cocultured with RA-FLS for 48 h in the presence of Transwell membrane barriers that permit only the diffusion of soluble factors. As shown in Fig. 3D, the synergistic increase in PlGF secretion during co-culture of MSC and FLS was completely abolished by the insert of a Transwell.

Increased angiogenesis and chemotaxis by cocultured medium of MSC and FLS

We next wanted to test if PlGF secreted into culture medium shows angiogenic activity and increases chemotaxis. To this end, an in vivo angiogenesis assay was performed by mixing Matrigel with CM from FLS alone (Fig. 4A). In addition, CM from cocultured FLS and MSC significantly increased chemotaxis of MSC more than CM from FLS alone (Fig. 4B). Moreover, the angiogenic and chemotactic effects of cocultured CM were significantly suppressed by preincubation of the CM with neutralizing anti-PlGF Ab (Fig. 4A, 4B), indicating that newly generated PlGF mediates CM-induced angiogenesis and chemotaxis. These data, together with the results of the Transwell experiment (Fig. 3D), suggest that PlGF produced via the interaction of FLS and MSC is biologically relevant and may contribute to chronic synovitis by increasing angiogenesis and chemotaxis of MSC.

Role of cadherin-11 in the interaction between FLS and MSC

Both ICAM-1 and VCAM-1, members of the Ig-like superfamily, are important for cell adhesion (27) and known to be expressed in RA-FLS (25) as well as in MSC (28). Inhibition experiments with blocking Abs against ICAM-1 and VCAM-1 were undertaken to determine whether these molecules are involved in cell-to-cell contact to enhance PlGF production; these Abs displayed a neutralizing activity against respective adhesion molecules in the preliminary binding experiment using THP-1 cells and HUVEC (data not shown). As shown in Fig. 5A, neither Abs against ICAM-1 nor VCAM-1 affected the production of PlGF during coculture of MSC and FLS.

Cadherins are known to mediate calcium-dependent adhesive interactions with the same cadherin species on a neighboring cell (29). Among the cadherin family, cadherin-11 is expressed predominantly on mesenchymal tissues (30). Engagement of cadherin-11 increases the expression of VEGF-D in mouse fibroblasts (31).
On the basis of the previous reports (29–31), we determined the involvement of cadherin-11 in the direct contact between FLS and MSC. Because cadherin action is dependent on calcium, we first tested the effect of EGTA, the extracellular calcium chelator, on PlGF production by coculture of FLS and MSC. As shown in Fig. 5B, PlGF production in the RA-FLS culture was suppressed by the addition of 1 mM EGTA, but this did not reach statistical significance (*p = 0.053). However, coculture-induced increase in PlGF secretion was dose-dependently repressed by the addition of EGTA. Next, we examined the constitutive and TGF-β–stimulated expression of cadherin-11 in RA-FLS and MSC by Western blot analysis. As seen in Fig. 5C, cadherin-11 was constitutively expressed in MSC and FLS, and its level was significantly increased by the addition of TGF-β (Fig. 5C). Finally, we performed a blocking experiment using cadherin-11 siRNA. Western blot analysis showed that transfection of FLS with cadherin-11 siRNA, but not control siRNA, curtailed the expression of cadherin-11 (inset, Fig. 5D). When RA-FLS were cocultured with MSC, cadherin-11 siRNA significantly cancelled the coculture-mediated increase in PlGF production (Fig. 5D). A similar degree of inhibition was observed when RA-FLS and MSC were cocultured in the presence of TGF-β.

**Discussion**

MSC have been reported to exist in the synovial membrane (12) and SF of patients with arthritis (13). Migration of MSC from the BM into the affected joints may represent a physiological response to the local tissue injury. Considering the previous observations that the influx of MSC in mice with collagen-induced arthritis is abolished by anti–TNF-α treatment (14), and immunosuppressive properties of MSC are reversed in the presence of TNF-α (15), the influx of MSC by a local inflammatory milieu to arthritic joints appears to contribute to synovial proliferation and joint destruction through autocrine and/or paracrine production of cytokines, chemokines, matrix metalloproteinases, and cell-cycle regulators (16, 32). However, factors involved in the chemotactic migration of MSC into the synovium and biological consequences of interactions between MSC and resident synoviocytes have not been clarified.

Activated fibroblast-like synoviocytes secrete a variety of soluble factors, and some of them affect the migration of MSC. PlGF, a member of the VEGF family, is highly expressed in the lining layer of hyperplastic RA synovium and is also increased in the SF of RA patients (5). In the current study, we showed that PlGF induced the migration of human BM-derived MSC in a dose-dependent manner. Unlike VEGF, PlGF is known to specifically bind VEGFR-1 (23). VEGFR-1 is expressed not only on vascular ECs but also on some non-ECs, including smooth muscle cells, monocytes, osteoblasts, and MSC (33–36). Likewise (36), we found that BM-derived MSC from four different donors constitutively expressed the VEGFR-1 at both the mRNA and protein levels, and stimulation with PlGF induced the tyrosine phosphorylation of VEGFR-1 (Fig. 1), indicating that PlGF transmits the distinct signals for MSC migration through VEGFR-1. This assertion is corroborated by the result showing that PlGF-induced chemotaxis was significantly suppressed by an anti–VEGFR-1
hexapeptide (GNQWFI). In addition, our findings are analogous to the previous data of Luttun et al. (37), who demonstrated that anti–VEGFR-1 Ab decreased the mobilization of BM-derived myeloid progenitors into the circulation and also inhibited the migration of VEGFR-1–expressing monocytes to the sites of inflammation.

Chemotaxis is a complex process in which an attractant binds to a specific membrane receptor, thus activating signal transduction pathways. PlGF induces the activation of multiple intracellular signaling protein including ERK1/2, p38 MAPK, JNK, PI3K/Akt, and stress-activated protein kinase in several cell types (7, 38, 39), and some of them have been implicated in MSC migration (40–42). Our data showed that PlGF-induced MSC chemotaxis was dependent on the activation of PI3K/Akt and p38 MAPK. Akt seems to act downstream of p38 MAPK in the MSC migration because Akt phosphorylation triggered by PlGF was abrogated by p38 MAPK inhibitor SB203580, whereas p38 phosphorylation was not altered by PI3K inhibitors LY294002 and wortmannin. Considering that Akt activation can influence cell motility through direct modulation of actin (43), this pathway may be a signaling hub that regulates the migration of MSC stimulated by PlGF. It is notable that defective migration of BM MSC from NOD mice is associated with suppression of the PI3K/Akt pathway, accompanied by the abnormal distribution of F-actin (44).

MSC are known to be recruited from the systemic circulation to the stroma of diverse growing tumors (45), which can be seen as resembling hyperplastic pannus tissues. Incorporated MSC promote tumor progression by interacting with tumor cells via direct cell contact and/or secretion of paracrine trophic factors. Indeed, the combined administration of MSC and tumor cells (B16-LacZ cells or LLC) promotes tumor growth in syngeneic tumor models partly through the enhancement of neovascularization (46). On the basis of these studies, we attempted to perform in vitro coculture assay using FLS and BM-MSC. We found that PlGF was constitutively expressed in FLS, but negligibly in MSC. However, coculture of FLS with BM-MSC induced a synergistic increase in PlGF secretion by FLS. The biological activity of secreted PlGF in the coculture supernatant was identified to be functional as demonstrated by the cocultured CM-induced increase in angiogenesis and chemotaxis of MSC, both of which were significantly inhibited by anti-PlGF Ab. These data underscore the importance of the MSC/FLS interaction in the augmented production of PlGF, confirming the proangiogenic activity of PlGF. In addition, considering that PlGF triggered MSC migration (Fig. 2), the data provide evidence for a positive-feedback loop for MSC migration that involves PlGF.

**FIGURE 4.** (A) CM obtained from coculture of RA-FLS and MSC enhances blood vessel growth in Matrigel plugs in vivo. C57BL/6 mice were injected s.c. with Matrigel containing CM from FLS culture (FLS-CM) and coculture of FLS and MSC (FLS/MSC-CM) as described in Materials and Methods. The representative pictures for hemoglobin content (*left panel*) and blood vessel formation (*right panel*; original magnification × 100) in the Matrigel containing CM of coculture are shown. Preincubation of FLS/MSC-CM with neutralizing anti-PlGF Ab (70 μg/ml) significantly decreased the hemoglobin content as well as blood vessel growth, *p < 0.005 versus FLS-CM and control medium (Con), **p < 0.05 versus FLS/MSC-CM without anti-PlGF Ab. Bars show the mean ± SD (n = 12). (B) CM of coculture increases chemotaxis of MSC. MSC were added to the upper wells of chemotaxis chamber, and FLS/MSC-CM, FLS-CM, and untreated control medium (Con) were added to the lower wells in the presence or absence of neutralizing anti-PlGF Ab (70 μg/ml), followed by the chemotaxis assay as described in Materials and Methods. The increased chemotaxis of MSC by cocultured CM was significantly suppressed by pretreatment with neutralizing anti-PlGF Ab (original magnification × 200), *p < 0.005, **p < 0.0005 versus Con, †p < 0.01 versus FLS/MSC-CM only, ‡p < 0.05 versus FLS-CM only, §p < 0.005 versus FLS-CM only. Bars show the mean ± SD of triplicate (n = 3).
Using a Transwell insert coculture system, cell-to-cell contact was shown to be indispensable for the synergistic production of PlGF by FLS. Additionally, depletion of extracellular Ca\(^{2+}\) ions by EGTA significantly decreased the production of PlGF, suggesting that calcium-dependent cell–cell interactions are required for PlGF upregulation. Among the cadherin superfamily members, cadherin-11 is known to mediate homophilic cell–cell interactions in a calcium-dependent manner (47). The role of cadherin-11 in RA synovitis was demonstrated by Chang et al. (48), who showed that engagement of cadherin-11 on FLS produced proinflammatory mediators including IL-6, MCP-1, IL-8, and macrophage migration inhibition factor. In our study, we observed that TGF-β increased the expression of cadherin-11 in both FLS and MSC. Interestingly, TGF-β-induced PlGF secretion was significantly suppressed when MSC was cocultured with cadherin-11 siRNA-transfected FLS. Considering that TGF-β failed to increase PlGF production without coculture (Fig. 3C), the TGF-β increase of PlGF production with coculture seems to be mediated, at least in part, by its upregulatory effect on cadherin-11 expression, as shown in Fig. 5D.

Notably, Matrigels with CM from FLS of OA patients and MSC coculture can be partly explained by the fact that staining scores of cadherin-11 expression in OA synovium were similar to those seen in RA synovium (49). Nevertheless, given the PlGF concentrations in SF (5) and its chemotactic activity for MSC (Figs. 2A, 4B), MSC-driven angiogenesis may not be vigorous in OA joints because the number of MSC that come into contact with FLS would be much lower in OA synovium than in RA synovium (50). Moreover, arthritic and aggressive RA synovium was demonstrated to contain a substantial (>30%) fraction of BM-derived precursors (16), which is considered to be crucial to the development of synovial hyperplasia in mice with chronic arthritis.

In summary, we showed that PlGF exerted migratory effects on human BM-derived MSC through the VEGFR-1 receptor. The PlGF-induced chemotactic response was dependent on p38 MAPK and Akt/PI3K signal pathways. Furthermore, coculture of MSC and FLS resulted in a synergistic increase in PlGF production, and such effects appeared to require MSC on FLS contact through cadherin-11. Taken together, these results suggest that MSC migrate into RA joints by the effect of PlGF, then interact with resident FLS in RA synovium via cadherin-11, and thereby exacerbate angiogenesis by further enhancing the production of PlGF.
Disclosures
The authors have no financial conflicts of interest.

References
Corrections


In Fig. 2, the incorrect β-actin bands were mistakenly included on the bottom row of the righthand panel of Western blots in Fig. 2C. There should have been five β-actin bands, but only four bands were shown. The corrected Fig. 2 is shown below. The figure legend was correct as published and is shown below for reference.

**FIGURE 2.** PlGF increases chemotaxis of MSC. MSC were added to the upper wells of the chemotaxis chamber. PlGF, medium alone (control [Con]), or VEGF was added to the lower wells. In the blocking experiments, anti–VEGFR-1 hexapeptide (Hexa; 10–80 μg/ml) was added to the upper wells. Chambers were then incubated for 12 h, and the migrated cells were counted [(A), left panel; original magnification ×200]. Results were expressed as a chemotactic index [(A), right panel]. The migrating cells (mean ± SD) in untreated control wells were 27 ± 5.5 cells/high-power field. *p < 0.01, **p < 0.001 versus Con, †p < 0.005 versus 10 ng/ml PlGF. (B) Suppression of MSC chemotaxis by inhibitors of p38 MAPK and PI3K. MSC were preincubated with SB203580 (SB; 10 μM), LY294002 (LY; 5 μM), PD98059 (PD; 50 μM), or SP600125 (SP; 25 μM) for 30 min and then treated with PlGF, followed by a chemotaxis assay. The migrating cells (mean ± SD) in untreated control wells were 23 ± 1.6 cells/high-power field. *p < 0.05 versus Con, **p < 0.005 versus 10 ng/ml PlGF only. (C) Activation of p38 MAPK and Akt by PlGF. MSC were preincubated in the presence or absence of LY, wortmannin (Wort), or SB, followed by PlGF treatment for 15 min. Cell lysates were resolved in SDS-PAGE and probed with Abs against total and p-p38 MAPK, p-ERK1/2, and p-Akt. Bars show the mean ± SD of three independent experiments (n = 3 donors). *p < 0.05 versus Con, **p < 0.005 versus PlGF only.