VE-cadherin-dependent vasculogenic mimicry-like tube formation in rheumatoid arthritic synovium

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Abstract

Increased vascularity in the synovium of patients with rheumatoid arthritis (RA) is thought to result from angiogenesis. However, other possibilities to develop vasculatures by mechanism(s) like vasculogenic mimicry remain elusive. In this study, expression of vascular endothelial (VE)-cadherin was immunohistochemically detected in both microvessel lining cells and fibroblastic cells in RA synovium. VE-cadherin-positive lining cells surrounded by periodic acid-Schiff staining-positive substance were occasionally negative for endothelial cell markers CD31/CD34 but positive for osteoblast (OB)-cadherin that is specific to fibroblastic cells. RA synovial fibroblast-like cells (RSFLs) in culture expressed VE-cadherin and OB-cadherin, but not CD31/CD34. Tube formation was evaluated by real-time adhesion and tube formation assays. RSFLs specifically bound to VE-cadherin-chimera-coated plates. They developed tube-like structures solely, which were hindered by anti-VE-cadherin antibody or VE-cadherin-chimera, and with endothelial cells. VE-cadherin was up-regulated by vascular endothelial growth factor-A_165 (VEGF_165) treatment, and shed from cell surface by treatment with tumor necrosis factor-a. RSFLs expressed VEGF and VEGF receptors (VEGFRs), and VEGF_165 induced up-regulation of VE-cadherin was suppressed by inhibiting VEGFR2 activity. These data demonstrate that fibroblastic cells in RA synovium express VE-cadherin, and suggest that the fibroblastic cells are involved in formation of vasculogenic mimicry-like tubes in RA synovium through the VEGF/VEGFR2/VE-cadherin pathway.

Introduction

Rheumatoid arthritis (RA) is one of the common and intractable systemic diseases, which progressively destroys diarthrodial joints. Normal synovium consists of the synovial lining cell layer, which is composed of synoviocytes including macrophage-like A cells and fibroblast-like B cells, and the sublining cell layer comprises fibroadipose tissue with sprinkled vasculatures. Increased vascular channels facilitate inflammatory cell infiltration and cytokine production, both of which are the initial and representative changes in RA synovium. These lead to fully developed chronic proliferative synovitis and pannus formation, contributing to cartilage and bone destruction in the joints. The increased vascular channels are formed in early phase of the disease and worsen the clinical outcome of the patients by supplying inflammatory cells, oxygen and nutrients, which make the synovium more complex structures and expand synovial tissue [1,2].

The vascular channel is considered to sprout from pre-existing blood vessels in a process of angiogenesis, which requires mutual endothelial cell binding through their intrinsic vascular endothelial (VE)-cadherin that controls vascular permeability [3,4]. In pathological states such as RA, vascular endothelial growth factor-A (VEGF), inflammatory cytokines and hypoxia are implicated in vascular channel formation. On the other hand, emerging lines of evidence disclosed that vasculogenic mimicry, which is characterized by immature channels made of VE-cadherin-expressing tumor cells, contribute to a progressive expansion of malignant tumors and correlate with poor prognoses of the patients [5-7]. Although vasculogenic mimicry is determined as specific in neoplastic diseases, Damsky's group showed VE-cadherin expression by cytotrophoblasts forming blood vessel-like vasculatures in placenta [8], and
recent studies indicates that it is indispensable to connect the vasculatures with maternal endothelial cell-lined blood vessels for nutrition and gas exchange of embryo [9]. These suggest the possibility that vascular channel formation in non-malignant tumor tissues can occur not only by angiogenesis but also other mechanisms. Synovial membrane is unique in that it can be completely repaired after tissue damages and contains the stem cell population [10,11]. A previous study showed the presence of immature microvascular channels in RA synovium that are frequently lined by cells morphologically different from typical endothelial cells [12], suggesting a possibility that the vasculature may not simply result from angiogenic pathway.

In the present study, the expression of VE-cadherin in RA synovium was detected in fibroblastic cells and microvascular lining cells, and the latter of which were occasionally negative for endothelial cell markers CD31/CD34. Therefore, we explored the possible involvement of synovial fibroblastic cells, which were isolated from RA synovial tissues, in tube formation with or without endothelial cells and regulation of VE-cadherin expression. Our data suggest that VE-cadherin-positive fibroblastic cells are involved in vasculogenic mimicry-like tube formation in RA synovium by the VEGF$_{165}$ stimulation.

**Materials And Methods**

**Synovial tissues and immunohistochemistry**

Arthritic synovial tissues from knee joints of the patients with RA ($n=8$) and normal synovium from hip joints of the patients with femoral neck fracture (normal control; $n=3$) were obtained by surgery at Keio University Hospital and used for the experiments. They were fixed with formalin and paraffin-embedded specimens were subjected to immunohistochemistry. The primary antibodies against VE-cadherin (LifeSpan Biosciences, Seattle, WA), CD31 (DAKO, Glostrup, Denmark) or CD34 (DAKO) and biotinylated secondary antibodies were used. After incubation with streptavidin-biotin-complex, the color was developed with 3,3′-diaminobenzidin tetrahydrochlorides. Non-immune rabbit IgG instead of the primary antibody were incubated with tissue sections as a negative control. To identify the extracellular matrix-associated vascular channels, tissue sections were proceed to periodic acid-Schiff (PAS) staining. Number of PAS-positive vessels containing red blood cells and lined with CD31/CD34-positive cells, CD31/CD34-negative cells or the mixture in RA and normal synovial tissues was counted under microscope. For double-immunofluorescent staining for VE-cadherin and osteoblast (OB)-cadherin (Invitrogen, Camarillo, CA), Opal 4-color Kit (PerkinElmer, Waltham, MA) was used and observed by a confocal laser-scanning microscope (LSM 700; Carl Zeiss, Oberkochen, Germany). Informed consent was obtained from the patients for the experimental use of the samples. The study protocol that compiled with the principles outlined in the Declaration of Helsinki was approved by the Ethical Committee Review Boards at Keio University and Nippon Dental University.

**Cell Cultures**
Rheumatoid synovial fibroblast-like cells (RSFLs) of the human were purchased from Health Science Research Resources Bank (Osaka, Japan; RSFL1, HT32251238; RSFL2, HT51213801; RSFL3, HT47768187; and RSFL4, HT42487497) or isolated from RA synovium (RSFL5-7) [13, 14]. RSFLs between 5–8 passages were used to neglect contamination of different cell-types and phenotypic alterations of the cells according to a previous study [15]. They were maintained in DMEM containing 10% fetal bovine serum (FBS) and passaged at 7.6 x 10^3 cells/cm^2 when they reached at subconfluent states. Human umbilical vein endothelial cells (HUVEC) and GFP-labeled HUVEC (GFP-HUVEC; Angio-Proteomie, Boston, MA) were cultured in Quick Coating Solution (Angio-Proteomie)-coating culture dishes in EGM-2 MV medium (Lonza, Basel, Switzerland) containing 5% FBS. For stimulation of RSFLs, they were cultured in 1% FBS or FBS-free medium containing 0.2% lactalbumin hydrolysate for 24 h, and then treated with VEGF_{165} (80 ng/ml; R&D Systems, Minneapolis MN) and/or tumor necrosis factor-α (TNF-α; 10 ng/ml; AbD Serotec, Oxford, UK) for 72 h. For the experiment under the hypoxic condition, RSFLs were cultured in 1% O_{2} for 72 h using Bionix hypoxic culture kit (Sugiyama-gen, Tokyo, Japan) or treated with 150 µM deferoxamine (Sigma-Aldrich, St. Louis, MO) for 48 h. To analyze VEGF receptor (VEGFR)-dependency of VE-cadherin expression, RSFLs cultured for 24 h in FBS-free medium were treated with placental growth factor 1 (PROSPEC, Ness-Ziona, Israel) or SU1498 (1 µM; Calbiochem, San Diego, CA) for 72 h. For knockdown experiments, RSFLs were transfected with short interfering RNAs (siRNAs) against neuropilin 1 (siNRP1; 75 nM, sc-36038; Santa Cruz Biotechnology, Santa Cruz, CA), VE-cadherin (siVE-CDH; 75 nM, L-003641) or a negative control siRNA (siCtrl: 100 nM D-001810-10-05; Dharmacon, Chicago, IL). Experimental protocols were reviewed and approved by the institutional review boards of Nippon Dental University and Keio University.

Immunocytochemistry

RSFLs and HUVEC were cultured on Lab-Teck Chamber II (ThermoFisher Scientific, Waltham, MA) until 24 h after reaching the confluency to mature cell-cell contacts, and fixed in 3.7% paraformaldehyde for 15 min at 23°C. After treatment with 0.1% Triton X-100 in PBS for 7 min, the cells were reacted with antibodies to VE-cadherin or epithelial (E)-cadherin (clone HECD-1; R&D Systems), followed by incubation with Alexa Fluor 546 anti-rabbit IgG (Invitrogen). They were observed by a confocal laser-scanning microscope.

Immunoblot

Immunoblot

Total proteins of RSFLs were prepared by homogenization in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40) containing proteinase inhibitors (Complete mini proteinase inhibitor cocktail, Roche, Mannheim, Germany) on ice. For isolation of membrane and cytoplasmic/nuclear fractions, RSFLs were treated with ProteoExtract Native Membrane Extraction Kit (Calbiochem) according to the company's instruction. To analyze the VE-cadherin ectodomain shedding,
proteins were precipitated from the culture media with 3.3% trichloroacetic acid and the samples were resolved by SDS-polyacrylamide gels. Proteins electrotransferred onto PVDF membranes were subjected to immunoblot using antibodies against CD31, CD34, neural (N)-cadherin (Invitrogen), OB-cadherin, VEGF (clone A-20; Santa Cruz Biotechnology), VEGFR1, VEGFR2, NRP1 (Cell Signaling), GAPDH (Ambion, Austin, TX) or β-actin (Sigma-Aldrich). For immunoblot of VE-cadherin, antibodies specific to the COOH-terminal region (clone D87F2; Cell Signaling, Danvers, MA: and sc-6458; Santa Cruz Biotechnology) or the NH₂-terminal region of VE-cadherin (sc-52751, Santa Cruz Biotechnology) were used. After incubation with secondary antibodies, the bands were detected by ECL Select (GE Healthcare, Buckinghamshire, UK) and visualized using EZ-Capture (ATTO, Tokyo, Japan). Intensity of the bands (n = 4) standardized by that of β-actin bands were quantified using ImageJ software (NIH, Bethesda, MD; http://imagej.nih.gov/ij).

Reverse Transcription (Rt)-pcr

Total RNAs isolated from RSFLs were reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen) with oligo-dT primer and applied for PCR amplification. VEGF₁₂₁ (452 bp), VEGF₁₆₅ (584 bp) and VEGF₁₈₉ (656 bp) were detected by RT-PCR using the primers (forward, 5'-CGAAACCATGAACCTTCTGTGCTG-3'; reverse, 5'-TCACCGCCTCGGCTTGTCAT-3'). For the detection of VEGF₂₀₆, a specific primer set (forward, 5'-GAAGGAGGAGGGCAGAATCATCACG-3'; reverse, 5'-TCCAGGGGCATTAGACAGCAGCG-3') was used.

Cell Adhesion Assay

RSFLs adhesion on culture plates was monitored using RTCA-DP xCELLigence (Roche), which can monitor the adhesion by measuring electronic impedance at the cell-sensor electrode interface integrated on the bottom of culture E-plate [16, 17]. Each plate was pre-coated with 20 µg/ml of VE-cadherin or E-cadherin Fc-chimeras (R&D Systems) for 3 h at 37°C and then incubated with DMEM containing 0.25% FBS for 1 h at 37°C. RSFLs were seeded on a plate at a density of 1.7x10³ cells/plate, and installed on the analyzer. Cell adhesion was monitored every 2 min for 5 h.

Tube Formation Assay

RSFLs (2.5x10⁵ cells/cm²) labeled with CellTracker Red CMTPX (ThermoFisher Scientific) were cultured in serum-free condition for 24 h on dishes coated with Matrigel (µ slide angiogenesis; ibidi, Nippon Genetics, Tokyo, Japan) and observed by a phase contrast microscope (LSM700, Carl Zeiss). For inhibition of branching morphology, RSFLs were cultured on the gels for 24 h in culture medium containing anti-VE-cadherin antibody (10 µg/ml; BD biosciences, Franklin Lakes, NJ), anti-E-cadherin antibody (10 µg/ml; R&D Systems), non-immune rat IgG (10 µg/ml; Vector Laboratories, Burlingame, CA), VE-cadherin Fc-chimera (20 µg/ml) or E-cadherin Fc-chimera (20 µg/ml), and treated with siVE-CDH (75 nM) or siCtrl (100 nM). The branching point numbers were quantitatively evaluated according to the
previous study [18, 19]. To determine tube-like formation by RSFLs in the presence of HUVEC, RSFLs labeled with CellTracker Red CMTPX and GFP-HUVEC were co-cultured on basement membrane matrices for 24 h and monitored by a confocal laser-scanning microscope. The data of tube-like structures were analyzed using Zen software (Carl Zeiss).

**Statistical analysis**

Data obtained by the tube assay were shown as mean ± SD. Results between the two independent groups were determined by the student’s *t*-test or Mann-Whitney *U* test. Comparisons among more than three groups were analyzed by Tukey-Kramer’s HSD or Steel-Dwass test. *P* values less than 0.05 were considered significant.

**Results**

**Expression of VE-cadherin in RA synovium and RSFLs**

By immunohistochemistry for VE-cadherin, both vascular channel lining cells and stromal cells including fibroblastic cells were positive in RA synovial tissue (Fig. 1a), while negligible staining was obtained with non-immune IgG (Fig. 1c). The VE-cadherin-positive stromal cells were localized mainly to the areas rich with microvascular channels, which were commonly lined by plump cells compared to thin endothelial cells of larger vessels (Fig. 1a). Such VE-cadherin-positive stromal cells and plump cells were negligible in normal synovium (Fig. 1b). VE-cadherin-positive vasculatures in RA synovial tissues were commonly surrounded by PAS-positive basement membrane components (Fig. 1d), which include laminin, type IV collagen and perlcan that can be produced by RA synovial fibroblastic cells [20–22]. Since mature endothelial cells and the progenitor cells express CD31 and CD34, respectively [23], we double-immunostained RA synovium with anti-CD31 and anti-CD34 antibodies and showed the frequent staining in vascular lining cells (Fig. 1e). However, some vasculatures were lined with CD31/34-negative cells or a mixture of positive and negative cells (Fig. 1e) in contrast to normal synovium where most vasculatures were stained for CD31/CD34 (Fig. 1f). Among PAS-positive vasculatures containing red blood cells, 58.2 ± 16.0% (mean ± S.D.) were CD31/34-positive, 17.1 ± 10.7% CD31/34-negative, and 24.6 ± 9.4% the mixture (Fig. 1g). Almost all of the vasculatures in normal synovium were CD31/34-positive (96.0 ± 10.7%). These findings suggest the possibility that lining cells of vascular channels in RA synovium contain VE-cadherin-positive non-endothelial cells as in malignant tumors [5].

To examine the VE-cadherin expression in fibroblastic cells, RSFLs isolated from RA synovial tissues of different donors were subjected to immunocytochemistry. We used HUVEC as a control in this study because they are a most characterized endothelial cells about the origin and regulation of VE-cadherin expression and function. As shown in Fig. 2a, VE-cadherin, but not E-cadherin, was localized mainly at the cell-cell boundaries of RSFLs in a discontinuous punctate pattern, which contrasts to the belt-like continuous staining in HUVEC. Immunoblot analysis confirmed the VE-cadherin expression in RSFLs, although they were lower than that in HUVEC (Fig. 2b and c). The expression was verified by
disappearance after the knockdown by siVE-CDH (Fig. 2b). Contamination of endothelial cells and inflammatory cells in our RSFLs was unlikely because these cells disappear by passages more than four times after isolation from RA synovial tissues [15]. In addition, CD31 and CD34 were negative in RSFLs, whereas HUVEC expressed CD31 (Fig. 2b) that confirmed a previously reported [24]. RA synovium contain bone marrow stem cells that may be able to differentiate into endothelial cells expressing VE-cadherin and CD31 upon the differentiation and CD34 in the precursor states [25]. It is known that synovial fibroblastic cells but not endothelial cells and bone marrow-derived cells express OB-cadherin [26]. All RSFLs examined but not HUVEC expressed OB-cadherin (Fig. 2b and c), indicating that RSFLs are intrinsic fibroblastic cells in the synovium not derived from bone marrow and contained endothelial cells. N-cadherin was comparably detected in RSFLs and HUVEC (Fig. 2b and c). VE-cadherin and OB-cadherin expression were confirmed by immunostaining of normal and RA synovial tissues (Fig. 2d). Vascular lining cells were positively stained for VE-cadherin and perivascular and mesenchymal cells for OB-cadherin in normal synovium, whereas vascular lining cells in RA synovium occasionally stained both cadherins. Although case numbers examined in this study were limited and further studies are needed to conclude, above results strongly imply that vasculatures in RA synovium may not simply result from angiogenesis.

**Binding Of Rsfls To Ve-cadherin**

In the following experiments, we principally used RSFL1 and/or RSFL4 between passage 5–8 because they were representative for VE-cadherin expression than RSFLs 2 and 3 (data not shown) and RSFLs 5–7 were passaged over 8-times from the isolation that frequently associate with phenotypic alterations of the cells [15]. Since VE-cadherin fulfills its function on cell surfaces, we first examined the subcellular localization of VE-cadherin in cell membrane or cytoplasmic/nuclear fractions, and found that VE-cadherin was exclusive in the cell membrane fraction (Fig. 3a). When the binding of RSFLs to cadherin chimera proteins was tested, RSFLs bound to the plates coated with VE-cadherin chimera but not E-cadherin chimera (Fig. 3b upper panel). RSFLs showed the morphology of spindle-shaped cells with filopodial extensions on the VE-cadherin-coated plates, whereas they exhibited the spherical aggregates on E-cadherin-coated plates (Fig. 3b lower panels).

**Ve-cadherin-mediated Tube-like Formation Of Rsfls**

We then evaluated tube formation activities of RSFLs. Since tube formation and activity are a very complex phenomena including cellular and extracellular regulation and their interactions [2, 18], it should be finally concluded by a series of *in vitro* and *in vivo* studies. This study used a widespread assay by culturing cells on Matrigel to quantify the VE-cadherin-dependence. Figure 3c shows representative well-developed tube-like structures by RSFLs without any treatment. We determined the VE-cadherin-dependence and quantified the activity by counting branching points indicative of initial reaction of the tube formation [18, 19, 27, 28]. The branching points appeared to be reduced by anti-VE-cadherin reagents.
but not anti-E-cadherin reagents. Quantification of the branching point number showed significant loss of the branching by inhibiting VE-cadherin (Fig. 3d).

**Tube-like Formation Of Rsfls With Huvec**

Since immunostaining data suggested that lining cells of the vasculature in RA synovium constitute by endothelial cells and fibroblastic cells, we tested this possibility by carrying out tube formation assay in a mixture of RSFLs and HUVEC in a 1:1 ratio. RSFLs were intermingled in the tubes with HUVEC (Fig. 4a, b, e and f), and the Z-stack and 3D image analyses showed an inclusion of RSFLs and HUVEC in the tubes and their direct contacts (Fig. 4c, d, g and h). These cells also developed similar structures in different ratios (RSFL:HUVEC; 1:3 and 3:1) (Fig. 4i-l).

**Ve-cadherin Expression By Angiogenic Factors**

When RSFLs were cultured in the presence of VEGF$_{165}$ or TNF-α or under hypoxia, all of which are stimulator for angiogenesis, intact VE-cadherin protein (125 kDa) expression appeared to be increased by VEGF$_{165}$ (Fig. 5a). Densitometric analysis of VE-cadherin protein bands confirmed a significant increase of VE-cadherin expression after the VEGF$_{165}$ treatment ($P=0.0495$) (Fig. 5b), and the cell membrane localization of VE-cadherin was not affected by the treatment (Fig. 3a). Treatment of RSFLs with TNF-α decreased VE-cadherin expression compared to the cells without treatment (0.28 ± 0.15) (Fig. 5c and d). Hypoxic condition under 1% O$_2$ increased the expression (1.68 ± 0.56), but an alternative hypoxic setting by deferoxamine treatment appeared to decrease the expression (Fig. 5c and d).

Upon treatment with TNF-α or hypoxic condition, RSFLs are reported to express ADAMs (a disintegrin and metalloproteinases) and MMPs (matrix metalloproteinases) that shed VE-cadherin protein of 125-kDa from cell surfaces and generate the 35-kDa cytoplasmic fragments [29, 30]. The 1% O$_2$ hypoxic condition facilitated production of both 125-kDa and 35-kDa forms (Fig. 5c and d). Unveiling a role of hypoxic condition on VE-cadherin expression is required further studies. Although the TNF-α treatment showing the 125-kDa form down-regulation did not associate with the 35-kDa fragment production (Fig. 5c and d), increase of 90-kDa VE-cadherin ectodomain shedding into the culture media was substantiated by the immunoprecipitation (Fig. 5e). Although co-treatment with VEGF$_{165}$ did not affect the 125-kDa and 35-kDa forms compared with TNF-α alone (Fig. 5c and d), enhanced expression of VEGF$_{165}$ and TNF-α in RA synovium may largely regulate VE-cadherin expression and shedding that result in a rapid turnover of VE-cadherin that accelerate angiogenesis [31, 32].

**Expression Of Vegf Isoforms And Vegfrs In Rsfls**

Fibroblastic cells in RA synovium are known to express VEGF showing a positive correlation with microvascular density [33]. VEGF consists of isoforms with different molecular weights, i.e. VEGF$_{121}$,
VEGF, VEGF, and VEGF, by alternative splicing of the gene transcript, and the dimer made of each isoform display different activities on angiogenesis [4]. Then, we profiled the expression of the isoforms in RSFLs and detected all the isoforms at both mRNA and protein levels, and the proteins were present as the dimers as well as the monomers (Fig. 6a and b). VEGF isoforms bind to the receptors including VEGFR1, VEGFR2 and NRP1, and VEGFR2 plays a dominant role to transmit VEGF signals. VEGFR1 antagonizes VEGF/VEGFR2 binding and NRP1 strengthens the binding, indicating that VEGFR1 and NRP1 suppress and enhance the signaling, respectively [4]. When VEGFR expression was examined by immunoblot, all receptors were detected in RSFLs (Fig. 6c). Down-regulation of 125-kDa VE-cadherin by TNF-α may not be attributed to inhibition of VEGFR2, since TNF-α increased NRP1 expression but not affect on VEGFR1 (Fig. 6d). Hypoxic condition under 1% CO2 did not change these receptor expression. VEGFR2 comparably expressed under the above conditions was shown as several bands (Fig. 6c) that may reflect the glycosylation states as reported previously [34], and the expression was confirmed by immunoprecipitation analysis (Fig. 6e). These results suggest that activation of VEGFR2 by the ligand binding regulates VE-cadherin expression.

Suppression Of Ve-cadherin Expression By Vegfr2 Inhibition

To assess whether the VEGF/VEGFR2 signaling affects VE-cadherin expression, we treated RSFLs with SU1498, an inhibitor of VEGFR2 kinase activity. Up-regulation of VE-cadherin by VEGF appeared to be inhibited by treatment with SU1498 as well as NRP1 siRNA (Fig. 6f and g). In addition, placental growth factor 1, a VEGFR1 agonist, exerted no influence on the expression (Fig. 6h). VEGF binding to VEGFR2 seems to be a key determinant for VE-cadherin expression in RSFLs.

Discussion

Increased microvasculature is one of the earliest and most prominent features in RA synovitis. Although vascular channel formation in RA synovium was ascribed to angiogenesis, the present study provided, to the best of our knowledge, the first evidence suggesting that the vasculature in RA synovium is generated, at least in part, by vasculogenic mimicry-like mechanisms through incorporation of VE-cadherin-positive endothelial cells and fibroblastic cells.

VE-cadherin/cadherin-5 is a member of the classical cadherin family and thought to be an endothelial specific adhesion molecule [35]. By locating at junctions between endothelial cells, VE-cadherin plays an essential role in the inter-endothelial cell contacts and maintenance of vascular integrity [36]. Concerning the expression of the cadherin family members, RSFLs were reported to express N-cadherin/cadherin-2 and OB-cadherin/cadherin-11, but not E-cadherin/cadherin-1 by immunoprecipitation analysis using anti-pan cadherin antibody [37]. Although no or little information about the expression of other cadherin molecules in RA synovium was available, our study has demonstrated the expression of VE-cadherin by fibroblastic cells and vascular lining cells in RA synovium, and also by cultured RSFLs. HT1080 fibrosarcoma cells spontaneously express VE-cadherin [38] and fibroblastic cells express VE-cadherin
when maintained in endothelial growth culture medium [39]. VE-cadherin expression may not be exclusive to endothelial cells but can be expressed in fibroblastic cell lineages under special conditions.

One of the essential functions of cadherin is providing cells with ability of homotypic cell-cell adhesion, and VE-cadherin-mediated adhesion is well known in endothelial cells [4, 35]. The present study has demonstrated that VE-cadherin-positive RSFLs selectively bind to VE-cadherin-coated plates, and disclosed that like HUVEC, RSFLs exhibit tube-like formation in VE-cadherin-specific manner during culture on Matrigel. Although previous studies on vasculogenic mimicry by tumors cells widely used tube formation assay on Matrigel, its activities as the tube should be carefully concluded by a series of in vitro and in vivo studies. However, we have verified that co-culture of VE-cadherin-positive RSFLs and HUVEC leads to the formation of mosaic tube-like structures composed of mixed cell types, and that vasculatures in RA synovial tissues were occasionally negative for endothelial cell markers (CD31 and CD34) and lined with VE-cadherin and OB-cadherin expressing cells by the immunostaining. OB-cadherin is specifically expressed by fibroblastic cell in the synovium [26]. Although detailed future analyses on tube function are needed, this study strongly suggests that synovial fibroblastic cells and endothelial cells form mosaic vessels in RA synovium.

Angiogenesis [1, 2] and vasculogenic mimicry [5, 6] are regulated by numerous growth factors, cytokines and other special tissue microenvironments. The present study examined the effects of VEGF_{165}, TNF-α and hypoxia on the VE-cadherin expression in RSFLs, and found that VEGF_{165} promotes the production of intact 125-kDa VE-cadherin, whereas TNF-α decreased it. The role of TNF-α is needed additional studies on the protein turnover to conclude, and the effect of hypoxia should be carefully evaluated because 1% O_2 condition increased 125-kDa VE-cadherin but another hypoxia condition by deferoxamine treatment decreased it.

Previous study on the expression of VEGF isoforms in RA and osteoarthritic synovia demonstrated that VEGF_{165} is predominantly expressed in RA synovium, localizing to macrophage-like synovial lining cells and sublining spindle-shaped cells, and also showed that the selective up-regulation of VEGF_{165} and its receptors VEGFR2 and NRP1 correlates with the increased vascular density in RA synovium [33]. Although the study did not identify cell type of the sublining spindle-shaped cells, this study showing the expression of all the VEGF isoforms including VEGF_{165} by RSFLs strongly suggests that fibroblastic cells in RA synovium are responsible, at least in part, for the expression for VEGF. In addition, our study on VEGFR in RSFLs suggests the possibility that synovial fibroblastic cells are stimulated to express VE-cadherin via the autocrine pathway of VEGF/VEGFR2. A most strong angiogenic factor, VEGF, dramatically accelerates tube-like formation by RSFLs, and inhibitors of VEGF-VEGFR2 binding and VEGFR2 activity suppresses VE-cadherin expression and tube-like formation in our recent study [19]. Altogether, these data lead a hypothesis of VEGF/VEGFR2 signaling-stimulation of vasculogenic mimicry-like tube formation in RA synovium that may be supported by VEGF/VEGFR2-dependent vasculogenic mimicry in malignant tumors [3, 6]. Accelerated turnover of VE-cadherin facilitates angiogenic process by promoting endothelial cell migration [31, 32], and hypoxia enhances migration of tumor cells and
formation of vasculogenic mimicry [40] and TNF-α tumor cell migration [41]. Although there are no previous studies defining a role of VE-cadherin turnover in vasculogenic mimicry, it seems likely that combined action of VEGF, TNF-α and hypoxia simulate formation of vasculogenic mimicry-like tubes in RA synovium.

Recent application of disease-modifying anti-rheumatic drugs using biological agents targeting the molecules involved in RA inflammation such as antibodies against TNF-α or IL-6 receptor has ameliorated outcome of the RA patients. However, the drugs are ineffective in 30–40% of the patients and uncertain in early phase of the disease [42, 43]. Joint cartilage destruction sometimes continue after remission of inflammatory reactions in the synovium was achieved by the drugs, suggesting involvement of non-inflammatory reactions in disease progression [44]. Additional new therapeutic strategies are required to improve the patients’ prognosis. Angiogenesis inhibitors are expected to be useful for RA treatment especially at the early phase, but many of them jeopardize patients in ischemic heart diseases that are basically increasing with RA [2, 45]. In addition, the clinical trials of drugs targeting angiogenesis are often ineffective in patients with malignant tumors, in which vasculogenic mimicry has been made [7]. Vasculogenic mimicry is considered to be regulated by different mechanisms from angiogenesis and resistant to anti-angiogenesis drugs [5, 6, 46, 47]. Synovial fibroblast-like cells are expected as therapeutic target of RA especially in the early stage [48]. The present study showing vasculogenic mimicry-like tube formation including fibroblast-like cells suggests a possibility that the inhibition is a remedy to treat patients with RA.

**Declarations**

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**Author contribution**

N. Y. and H. S. performed experiments and analyzed the data. Y. T. and Y. S. performed experiments. H. M. and Y. O. provided critical materials. K. I. analyzed the data, designed and managed the project, and wrote the paper. All authors discussed the results and approved the final manuscript.

**Competing interests**

The authors declare no competing interests.

**Data availability**

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.
References


Figures
Figure 1

VE-cadherin expression by endothelial cells and fibroblastic cells in RA synovium. (a) Immunostaining of VE-cadherin. RA synovium was immunostained with anti-VE-cadherin antibody. Boxed areas are shown at higher magnification to the right. Arrowheads and arrows indicate VE-cadherin-positive plump endothelial cells of the microvascular channels and VE-cadherin-positive fibroblastic cells, respectively. *shows larger blood vessels that are covered by thin endothelial cell. (b) Immunostaining of normal synovium with anti-
VE-cadherin antibody. (c) RA synovium immunostained with non-immune IgG as a negative control. (d) RA synovium immunostained with anti-VE-cadherin antibody, followed by PAS staining. (e) RA synovium double-immunostained for CD31 and CD34, followed by PAS staining. White and black arrowheads show microvascular channels covered by CD31/CD34-positive and -negative cells, respectively. (f) Normal synovium stained for CD31/CD34 followed by PAS staining. Scale bars, 40 µm. (g) Percentage of vasculatures covered by CD31- and CD34-positive cells (+), -negative cells (-) or the mixture (+/-) in the synovium of normal (430 vessels in 3 cases) and RA joints (1,924 vessels in 8 cases). The data are presented as means ± SD. *, P < 0.05.
Figure 2

Cadherin expression in RSFLs and synovial tissues. (a) Fluorescent immunostaining for VE-cadherin and E-cadherin in RSFL4 (VE/RSFL and E/RSFL, respectively) and VE-cadherin in HUVEC (VE/HUVEC). Arrowheads indicate the discontinuous punctate staining in RSFLs. Scale bars, 10 µm. (b) VE-cadherin (VE-CDH), CD31, CD34, OB-cadherin (OB-CDH) and N-cadherin (N-CDH) expression in RSFLs and HUVEC. Cell lysates of RSFLs (RSFL1, 4, 5 and 7) were subjected to immunoblot with these antibodies. HUVEC
lysates loaded for VE-CDH immunoblot was one tenth of RSFL samples. RSFL4 treated with siRNA specific to VE-CDH (siVE) or non-silencing control siRNA (siCtrl) was subjected to the immunoblot for VE-CDH. β-actin, loading control. (c) Protein band densities for VE-CDH, OB-CDH and N-CDH standardized by the expression in HUVEC. (d) Immunostaining of VE-CDH (red) and OB-CDH (green) in normal synovium (left panel) and RA synovium (right panel). Arrowheads show vascular lining cells positive for VE-CDH and OB-CDH. Scale bars, 40 µm.
Membrane expression of VE-cadherin in RSFLs, binding of RSFLs to VE-cadherin and formation of tube-like structures. **(a)** Expression of VE-cadherin (VE-CDH) in the cell membrane fraction but not cytoplasmic fraction of RSFL4 with or without VEGF treatment. OB-cadherin (OB-CDH) and GAPDH were used as positive controls for membrane and cytoplasmic fractions, respectively. **(b)** Real-time monitoring of RSFL4 binding on VE-CDH Fc-chimera (VE-CDH Fc) and E-cadherin Fc-chimera (E-CDH Fc). Normalized cell index (CI) indicates the binding. Representative morphologic appearance of RSFL4 on VE-CDH Fc- and E-CDH Fc-coated plates is shown in the lower panel. Arrowheads, the filopodial extensions of RSFL. Scale bars, 80 µm. **(c)** Formation of tube-like structure by RSFL4. Ctrl shows RSFL4 cultured for 24 h on Matrigel. Branching structures of cells cultured in the media containing non-immune IgG (IgG), anti-VE-CDH antibody (anti-VE), anti-E-CDH antibody (anti-E), VE-CDH Fc (VE-Fc), E-CDH Fc (E-Fc), and transfected with siVE-cadherin (siVE) or negative control siRNA (siCtrl). Scale bars, 80 µm. **(d)** Evaluation of branching points of RSFL4 in each condition. Numbers were plotted as mean ± S.D. *, $P < 0.05$ compared to Ctrl ($n = 3$).
Figure 4

Tube formation in a mixture of RSFL and HUVEC. RSFL4 and GFP-HUVEC were seeded in a 1:1 ratio and cultured for 24 h (a-h). (a and e) Phase contrast micrographs of tube formation. (b and f) Immunofluorescent micrographs of the cells in bright field. RSFL4 were labeled in red and GFP-HUVEC in green. Boxed areas are shown at higher magnification (e and f) (c and g) Immunofluorescent micrographs of the cells by analyzing at the x-z stack (orange line) and y-z stack (blue line). Each stack was obtained from 16 slices at 20 µm intervals. (d and h) 3D imaging of the cell distribution. Tube formation by RSFL4 and GFP-HUVEC seeded in 1:3 (i and j) and 3:1 (k and l) ratios. Scale bars, 100 µm (a-c and i-l) and 15 µm (e-g).
Figure 5

Protein expression of VE-cadherin species in RSFLs under the simulation. (a) Immunoblot of 125-kDa intact and 35-kDa cytoplasmic fragment of VE-cadherin (VE-CDH) upon VEGF$_{165}$ treatment. (b) Densitometric analysis of the intact VE-cadherin bands in RSFL4 treated with (+) or without (-) VEGF$_{165}$ (n = 3). The band density was standardized by that of β-actin bands, and presented as means ± S.D. *, P < 0.05. (c) Immunoblot of intact and cytoplasmic fragment of VE-CDH in RSFL4 after treatment with cytokines or under hypoxia. The cells were treated with TNF-α in the absence or presence of VEGF$_{165}$ and under hypoxic conditions of 1% O$_2$ or deferoxamine treatment (DFX). Ctrl, control without treatment. (d) Protein band densities of VE-CDH (125 kDa and 35 kDa) in RSFL4 compared with Ctrl RSFL4. (e) VE-
cadherin ectodomain (90-kDa) in culture medium after TNF-a treatment. RSFL4 treated with or without TNF-a were precipitated by trichloroacetic acid and detected with anti-VE-cadherin antibody.

**Fig. 6**

(a) Expression of VEGF isoforms and VEGF receptors in RSFLs and changes in their expression under various conditions. (a) Expression of VEGF_{121}, VEGF_{165}, VEGF_{189} and VEGF_{206} mRNA detected by RT-PCR in RSFL1 and RSFL4. RT-PCR was carried out with (+) or without (-) reverse transcription (RT). (b) Immunoblot of VEGF protein isoforms in RSFL4. Cell lysates were subjected to immunoblot with anti-VEGF antibody. (c) Expression of VEGFRs including NRP1, VEGFR1 and VEGFR2 in RSFLs under treatment with or without TNF-a or hypoxia (1% O_2). (d) Protein band densities of NRP1 and VEGFR1 compared to control cells (n = 4). (e) Immunoprecipitation of VEGFR2 from RSFL lysates. They were immunoprecipitated with anti-VEGFR2 antibody (IP: anti-VEGFR2) or non-immune IgG (IP: Ctrl-IgG), and probed with anti-VEGFR2 antibody (WB: anti-VEGFR2). VEGFR2 expression in whole lysates of RSFL1, 3 and 4 was shown. (f) VE-cadherin expression in RSFL4 after treatment with SU1498 (SU) or siNRP1. (g) VE-CDH protein band densities with or with treatment of SU or siNRP1 normalized by control cells (n = 3). **, P < 0.01. (h) VE-cadherin expression in RSFL4 after treatment with placenta growth factor (PIGF).