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Endothelial Precursor Cells in the Synovial Tissue of Patients With Rheumatoid Arthritis and Osteoarthritis

Beate Rüger, Alexander Giurea, Axel H. Wanivenhaus, Harald Zehetgruber, David Hollemann, Genya Yanagida, Marion Groger, Peter Petzelbauer, Josef S. Smolen, Paul Hoecker, and Michael B. Fischer

Objective. **To find evidence for the presence of endothelial precursor cells, which can induce new vessel formation, in the synovial tissue of patients with rheumatoid arthritis (RA) and osteoarthritis (OA).**

Methods. **Precursor cells in the synovial tissue of 18 RA patients and 15 OA patients were identified by immunohistochemistry, morphometric analysis, and confocal laser scanning microscopy using the following phenotype markers: CD31, CD34, STRO-1, CD133, vascular endothelial growth factor receptor 2 (VEGFR-2), and CXCR4. The presence of CD31, CD34, CD133, VEGFR-2, and CXCR4 messenger RNA in the synovial tissue was determined by reverse transcriptase–polymerase chain reaction, and the message for CXCR4 was quantified by an RNase protection assay.**

Results. **A population of cells that expressed CD34 on their surface but lacked the endothelial cell marker CD31 was found in the synovial tissue of RA and OA patients. CD34+,CD31− cells were detected in close proximity to STRO-1+ and CD133+ cells, forming cell clusters in the sublining area of the synovial membrane. Within these cell clusters, CD34+,CD31− precursor cells were located on the inside surrounded by STRO-1+ cells and with CD133+ cells on the outside. CD34+ precursor cells in the cell layer expressed high levels of the chemokine receptor CXCR4, while VEGFR-2 was expressed on CD34+ and CD133+ cells, and α-smooth muscle actin was expressed on STRO-1+ cells.**

Conclusion. **The presence of endothelial precursor cells in the synovial tissue of RA and OA patients provides evidence for vasculogenesis induced by precursor cells that arise in situ or from circulating progenitors.**

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, characterized by destruction of multiple joints in which the articular cartilage and bone are destroyed by proliferative synovitis. The synovial lesion is formed by proliferation of synovial lining cells, thickening of the synovial membrane with villous projections, dense infiltration of the synovia with lymphocytes, macrophages, dendritic cells, and plasma cells, and the presence of osteoclasts (1). Osteoarthritis (OA) is considered to be a degenerative disease of the hyaline articular cartilage related to mechanical damage and aging. Inflammatory infiltration of the synovial membrane with lymphoid cells is found in a proportion of OA patients (2). Loss of the perichondrium and fibrillation of the cartilaginous matrix lead to eburnation of the bone surface and deformation of the joint. Although the pathophysiologic mechanisms of RA and OA have been described extensively, the development of endothelial precursor cells in the inflamed and damaged joint along the endothelial lineage is still an enigma (3,4).

In the peripheral blood, circulating bone marrow–derived stem cells represent <0.01% of nucleated cells, and phenotypically distinct populations of stem cells can be found (5). The majority of stem cells express the surface molecules CD34, CD133, CD117 (c-Kit), and CD90 (Thy-1) (6–8). Within the circulating CD34+ stem cell population, a subset expresses the endothelium-specific marker vascular endothelial

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Beate Rüger, PhD, Alexander Giurea, MD, Axel H. Wanivenhaus, MD, Harald Zehetgruber, MD, David Hollemann, PhD, Genya Yanagida, PhD, Marion Groger, PhD, Peter Petzelbauer, MD, Josef S. Smolen, MD, Paul Hoecker, MD, Michael B. Fischer, MD: University of Vienna, Vienna, Austria.

Address correspondence and reprint requests to Michael B. Fischer, MD, Department of Blood Group Serology and Transfusion Medicine, University of Vienna, AKH Ebene 4i, Währingergürtel 18-20, 1090 Vienna, Austria. E-mail: michael.b.fischer@meduniwien.ac.at.

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growth factor receptor 2 (VEGFR-2) (9,10). These cells are considered circulating endothelial precursor cells or hemangioblasts, and they give rise to endothelial colonies in culture.

CD34+,VEGFR-2+ endothelial precursor cells represent <2% of the CD34+ cell population in the peripheral blood (9,10). In peripheral tissues, precursor cells reside in small niches involved in the permanent restoration of damaged tissue, since continuous self-renewal requires the presence of tissue-specific progenitor cells (11). They are recruited from niches to differentiate to locally required mature cell types, and they can be replaced by circulating CD34+ stem cells which are in a "steady state" of equilibrium with resident precursor cells in the niches. It has been speculated that circulating endothelial progenitor cells expressing CD34 and VEGFR-2 can migrate to the inflamed joint, be incorporated into pathologic neovascular foci, and induce vasculogenesis (3,12). CD34+,VEGFR-2+ cells expressing CD133 have the greatest in vitro potential to differentiate along the endothelial lineage to more mature endothelial cells (11,13). During maturation, these cells lose CD133 but still express CD34 and VEGFR-2.

New vessels can also be generated by angiogenesis, the sprouting of new capillaries from preexisting vessels, which does not require endothelial precursor cells from the circulation (14,15). To allow subsequent sprouting, mature vessels are first destabilized (14,15). New vessels formed by sprouting are initially immature and must develop further through remodeling and maturation. The process of blood vessel formation, growth, and stabilization is regulated by factors, such as the different VEGF isoforms, placental growth factor, angiopoietins, ephrins, transforming growth factor β, and platelet-derived growth factor, which must be very precisely orchestrated in terms of time, space, and dose to form a functional vascular network (16). Further, to ensure adequate vessel stabilization, a coordinated growth of endothelial cells, pericytes, smooth muscle cells, and fibroblasts is required (17,18). Subendothelial pericytes and smooth muscle cells can be generated from bone marrow stromal cells that coexpress STRO-1 and α-smooth muscle actin (α-SMA) (19,20). Under physiologic conditions, blood vessel formation is mandatory for remodeling of joint lesions, since cell regeneration, function, and survival depend on oxygen and nutrient supply, and virtually all cells must reside within 100 μm of a capillary (12,14,15).

In the present study, we investigated endothelial progenitor cells that arise in situ or from circulating precursors, inducing vasculogenesis in the synovial membrane of RA and OA patients. We also investigated their local differentiation along the endothelial lineage to more mature endothelial cells.

MATERIALS AND METHODS

Patients. Synovial tissues from 18 RA patients and 15 OA patients were obtained at the time of surgery for arthroplasty or synovectomy, after informed consent was obtained. Patients with RA or OA fulfilled the respective classification criteria (21,22). Synovial tissues from 11 joints from 3 organ donors were obtained in accordance with the European Society for Organ Transplantation and with the Austrian Hospital Act (KAG 1982, §62 a, b, c).

Immunohistochemistry, morphometric analysis, and confocal laser scanning microscopy. Serial sections (4 μm) of snap-frozen synovial membrane samples from patients with RA and OA were prepared, and immunohistochemistry analysis was performed as previously reported (23), with minor modifications. Acetone-fixed sections were incubated overnight at 4°C with the following mouse monoclonal antibodies diluted in Tris buffered saline (TBS)/1% bovine serum albumin (BSA): anti–STRO-1 (2.5 μg/ml; Developmental Studies Hybridoma Bank, Iowa City, IA), anti-CD133 (2.5 μg/ml [Miltenyi Biotec, Bergisch Gladbach, Germany] and 3 μg/ml [R&D Systems, Minneapolis, MN]), anti-CD34 (0.5 μg/ml [Immunotech, Marseilles, France] and 2.5 μg/ml [Becton Dickinson, San Jose, CA]), anti-CD31 (3.5 μg/ml; DakoCytomation, Carpinteria, CA), anti–VEGFR-2/fetal liver kinase 1/kinase insert domain receptor (2.5 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), anti–CXCR4/Fusin (2.5 μg/ml; PharMingen, San Diego, CA), anti–von Willebrand factor (anti-vWF) (2.5 μg/ml; DakoCytomation), and anti–muscle-specific actin α and γ (HHF-35) (1.5 μg/ml; Enzo Diagnostics, New York, NY).

The reactivity of primary antibodies was revealed using biotinylated sheep $F(ab')_2$ anti-mouse Ig (10 μg/ml; An-der-Grub, Kaumberg, Austria) diluted in 500 μg/ml normal human Ig (Intraglobin F; Biotest, Dreieich, Germany) followed by streptavidin–horseradish peroxidase (HRP) complex (20 μg/ml) (Extravidin-HRP; Sigma, St. Louis, MO). For the detection of STRO-1, CD133, VEGFR-2, and CXCR4, a supersensitive streptavidin–HRP conjugate (Biocare Medical, Walnut Creek, CA) was used. Endogenous peroxidase was blocked during the incubation period with secondary antibody by adding D-glucose and glucose oxidase type VII. The primary antibody was omitted for control purposes, and isotype controls were included in the protocol. The sections were exposed to 3-amino-9-ethylcarbazole (AEC; Sigma) and counterstained with Mayer's hemalum.

For 2-color immunolabeling, synovial tissues were incubated with monoclonal antibodies to CD133 (2.5 μg/ml), STRO-1 (2.5 μg/ml), and VEGFR-2 (2.5 μg/ml), followed by biotinylated sheep $F(ab')_2$ anti-mouse Ig and a supersensitive streptavidin–HRP conjugate. Endogenous peroxidase was blocked as described above, and antibody binding was visualized by exposure to AEC. Tissue sections were counterstained with fluorescein isothiocyanate (FITC)–conjugated anti-CD34 (5 μg/ml; Becton Dickinson) followed by a sequential incubation step with anti-FITC monoclonal antibody coupled to

alkaline phosphatase (300 milliunits/ml; Roche Diagnostics, Mannheim, Germany) and exposed to naphthol-AS-MX phosphate–Fast Blue BB (Sigma). Morphometric analysis was performed by counting the number of CD31+, CD34+, CD133+, and STRO-1+ cells as well as the total number of cells in ten 1-mm^2 fields selected at random; morphometric analyses were performed independently by two investigators.

For immunofluorescence studies, acetone-fixed synovial tissue sections (7 μm) were prepared as described above, incubated overnight at 4°C with anti-CD133 (2.5 μg/ml) diluted in TBS/1% BSA, subsequently blocked with 500 μg human IgG, and incubated with tetramethylrhodamine isothiocyanate (TRITC)–conjugated goat anti-mouse $F(ab')_2$ antibody (1 μg/ml; Jackson ImmunoResearch, West Grove, PA). Tissue samples were then blocked with mouse serum (1:50) and incubated with anti-CD34–FITC (5 μg/ml) and anti–STRO-1 (5 μg/ml) overnight at 4°C. Staining of the mouse IgM monoclonal anti–STRO-1 antibody was revealed using a biotin-conjugated goat anti-mouse IgM (2.5 μg/ml; Sigma) followed by a streptavidin–Cy5 complex (2 μg/ml; Jackson ImmunoResearch). Serial dilutions of each primary and secondary antibody were performed to minimize nonspecific binding, assure separation of the fluorescent signals, and optimize fluorophore concentration to preclude self-quenching. Sections were analyzed with a confocal laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany) with multiphoton laser (argon laser: 488 nm for FITC; HeNe 1: 543 nm for TRITC; HeNe 2: 633 nm for Cy5) and a 63 Zeiss Plan-Apochromat differential interference contrast oil immersion objective with numerical aperture 1.40.

Reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA was isolated from snap-frozen synovial tissues of patients with RA and OA, using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Subsequently, contaminating genomic DNA was removed by DNase I treatment using the DNA-free kit (Ambion, Austin, TX).

Equal amounts (3 μg) of total RNA, quantified at 260 nm, were reverse transcribed into complementary DNA (cDNA) using the SuperScript preamplification system for first-strand cDNA synthesis (Life Technologies) as described previously (23). The cDNA (1/20 of reverse-transcribed RNA for all primer pairs except for CD133, where 1/4 was used) was amplified in a gradient thermocycler (Hybaid; PCR Express, Ashford, Middlesex, UK) using *Taq* DNA polymerase (Life Technologies) and intron-spanning oligonucleotide primer pairs specific for CD31 (platelet endothelial cell adhesion molecule 1; sense 5′-TCC-AGT-GTC-CCC-AGA-AGC-3′ and antisense 5′-CAA-GGG-AGC-CTT-CCG-TTC-3′), CD34 (sense 5′-CTT-GCT-GAG-TTT-GCT-GCC-TTC-3′ and antisense 5′-ACA-TTT-CCA-GGT-GAC-AGG-CTA-3′), CD133 (sense 5′-TAC-CAA-GGA-CAA-GGC-GTT-CAC-3′ and antisense 5′-CAG-TCG-TGG-TTT-GGC-GTT-GTA-3′), CXCR4 (sense 5′-TTC-TAC-CCC-AAT-GAC-TTG-TG-3′ and antisense 5′-ATG-TAG-TAA-GGC-AGC-CAA-CA-3′), and VEGFR-2 (sense 5′-CAT-GTG-GTC-TCT-CTG-GTT-GTG-3′ and antisense 5′-TCC-CTG-GAA-GTC-CTC-CAC-ACT-3′).

The integrity of the RNA was checked using the low-expressing housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT; sense 5′-TGA-AAA-GGA-CCC-CAC-GAA-3′ and antisense 5′-ACA-ACA-ATC-CGC-CCA-AAG-G-3′). The amplification profile involved 35 cycles of denaturation at 94°C for 20 seconds, primer annealing for 30 seconds at 57°C for CD31, 59°C for CD34 and CD133, 60°C for VEGFR-2, and 55°C for CXCR4 and HPRT, and primer extension at 72°C for 45 seconds with a final extension step of 10 minutes. Ten-microliter aliquots of the PCR-generated products were resolved by electrophoresis on 2% agarose gels in Tris–acetate–EDTA buffer, exposed to ultraviolet (UV) light, and photographed using the GelDoc 2000 Documentation System (Bio-Rad, Richmond, CA). The predicted sizes of the PCR products were 462 bp for CD31, 364 bp for CD34, 449 bp for CD133, 206 bp for CXCR4, 251 bp for VEGFR-2, and 390 bp for HPRT. To control genomic contamination, an identical parallel PCR for CXCR4 was performed using RNA samples that had not been reverse transcribed, since chemokine receptor genes lack intron sequences.

Nonradioactive RNase protection assay. Chemokine receptor message expression was semiquantified with a nonradioactive RNase protection analysis system (RiboQuant; PharMingen). The hCR6 multiprobe template set was used for in vitro transcription reactions using T7 polymerase to direct synthesis of high-specific-activity biotin-labeled antisense RNAs that hybridize to human RNAs encoding CXCR1, CXCR2, CXCR3, CXCR4, Burkitt's lymphoma receptor 1 (BLR-1), BLR-2, V28, and two housekeeping gene products, L32 and GAPDH.

Templates were transcribed using a biotin RNA labeling mix (Roche Diagnostics). Twenty micrograms of total RNA from synovial tissues isolated with the TRIzol reagent (see above) was hybridized to the biotin-labeled hCR6 template set for 18 hours at 56°C and subsequently subjected to digestion with RNases A and T1, proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation. The samples were then run on a precast polyacrylamide 5% Tris–borate–EDTA–urea Criterion gel in a Criterion Cell (Bio-Rad, Hertfordshire, UK), blotted onto a positively charged nylon membrane (Roche Diagnostics) using a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad), and subsequently UV-crosslinked (Stratalinker 1800; Stratagene, La Jolla, CA). Bound biotinylated probe was then detected using the North2South Chemiluminescent Detection kit (Pierce, Rockford, IL), exposed for 15 minutes to Hyperfilm ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK), and photographed using the GelDoc 2000 Documentation System.

Statistical analysis. Student's *t*-test was used to evaluate differences in the number of cells/mm^2 in the synovial tissue of RA and OA patients, obtained by morphometric analysis. *P* values less than 0.05 were considered significant.

RESULTS

Expression of CD34, STRO-1, CD133, CXCR4, and VEGFR-2 on precursor cells in the synovial tissue of RA and OA patients. Morphometric analysis of synovial membranes of 15 RA patients gave a mean ± SD total of 702.1 ± 266.8 cells/mm^2, 12.4 ± 3.1 CD31+ cells/mm^2, 16.8 ± 5.0 CD34+ cells/mm^2, 11.5 ± 3.3

Table 1. Morphometric analysis of cells in the synovial tissue of patients with rheumatoid arthritis (RA) and osteoarthritis (OA)

	Cells/mm^2, mean ± SD				
	Total	CD31+	CD34+	CD133+	STRO-1+
Patients					
RA (n = 15)	702.1 ± 266.8*	12.4 ± 3.1	16.8 ± 5.0	5.3 ± 2.5*	11.5 ± 3.3
OA (n = 15)	230.8 ± 77.3	7.8 ± 2.2	9.0 ± 1.2	1.6 ± 1.2	7.3 ± 2.6
Healthy controls (n = 11)†	128.2 ± 13.4	6.9 ± 1.9	7.5 ± 2.2	0.2 ± 0.1	2.4 ± 1.3

* $P < 0.05$ versus OA patients.
† Synovial tissues from 11 joints from 3 organ donors.

STRO-1+ cells/mm^2, and 5.3 ± 2.5 CD133+ cells/mm^2 (Table 1). Synovial tissues of 15 OA patients showed a mean ± SD total of 230.8 ± 77.3 cells/mm^2, 7.8 ± 2.2 CD31+ cells/mm^2, 9.0 ± 1.2 CD34+ cells/mm^2, 7.3 ± 2.6 STRO-1+ cells/mm^2, and only 1.6 ± 1.2 CD133+ cells/mm^2, whereas in synovial membranes of healthy controls we found a mean ± SD total of 128.2 ± 13.4 cells/mm^2, 6.9 ± 1.9 CD31+ cells/mm^2, 7.5 ± 2.2 CD34+ cells/mm^2, 2.4 ± 1.3 STRO-1+ cells/mm^2, and only 0.2 ± 0.1 CD133+ cells/mm^2 (Table 1).

In the synovial tissue of RA and OA patients, the number of cells staining positive for CD34 was higher than the number of cells expressing the endothelial cell marker CD31 and vWF (data not shown). This discrepancy exists because CD34 is expressed on mature endothelial cells as well as on hematopoietic progenitor cells with the potential to differentiate to vascular endothelial cells. Figure 1 shows serial sections of synovial tissues of 1 representative RA patient and 1 representative OA patient. CD34+ cells that did not express CD31 and vWF, STRO-1+ cells, and CD133+ endothelial precursor cells were found in close proximity to small vessels within the sublining cell area of the synovial tissue of both RA and OA patients (Figure 1). They formed cell clusters, with CD34+ cells located in the center of the cluster (Figure 1A, inset) surrounded by STRO-1+ cells (Figure 1C, inset) and with CD133+ cells on the outside (Figure 1E, inset). Within this cell cluster, CD34+ cells expressed high levels of the chemokine receptor CXCR4 (Figure 1G, inset), while VEGFR-2 was equally expressed on single CD34+ and single CD133+ cells (Figure 1I, inset). The majority of STRO-1+ cells in the synovial tissue of RA and OA patients did not show CXCR4 on their cell surfaces but expressed low levels of VEGFR-2 and high levels of α-SMA (results not shown). In addition to expression on CD34+ precursor cells, CXCR4 was expressed on activated T cells in lymphoid aggregates in RA synovium.

Double immunolabeling extended these findings by showing that single CD133+ cells in the vessel and within the perivascular area did not express high levels of CD34 (Figures 2A and B), and STRO-1+ cells surrounding CD34+ cells were CD34− (Figure 2C). Further, the majority of CD34+ cells expressed VEGFR-2 on their surfaces (Figure 2D). Immunohistochemistry, however, cannot distinguish between high and low expression of these antigens. Three-color laser scanning microscopy was used to better characterize the expression pattern of CD34, CD133, and STRO-1 on these precursor cells in the synovial tissue. This technique allows for a better segregation of high and low CD34, CD133, and STRO-1 expression. Precursor cells in the center of the cell cluster expressed high levels of CD34 (Figures 3A and B), low levels of CD133 (Figures 3B and C), and no STRO-1 (Figures 3B and D). CD34+ cells were surrounded by cells expressing high levels of CD133 and low levels of CD34, and the majority of STRO-1+ cells on the outside of the cell cluster were CD34−. Interestingly, some of the STRO-1+ cells expressed low levels of CD133, which was not seen by immunohistochemistry analysis.

RT-PCR analysis of CD31, CD34, CD133, CXCR4, and VEGFR-2, and quantification of CXCR4 messenger RNA (mRNA) by an RNase protection assay in synovial tissues of RA and OA patients. Extraction of total RNA from synovial tissue samples from RA patients resulted in ~3–5-fold higher yields compared with samples from OA patients (data not shown). This corresponded to the higher number of total cells/mm^2 found in RA tissues due to lymphocyte infiltration (Table 1). Message for CD31 and CD34 was found in the

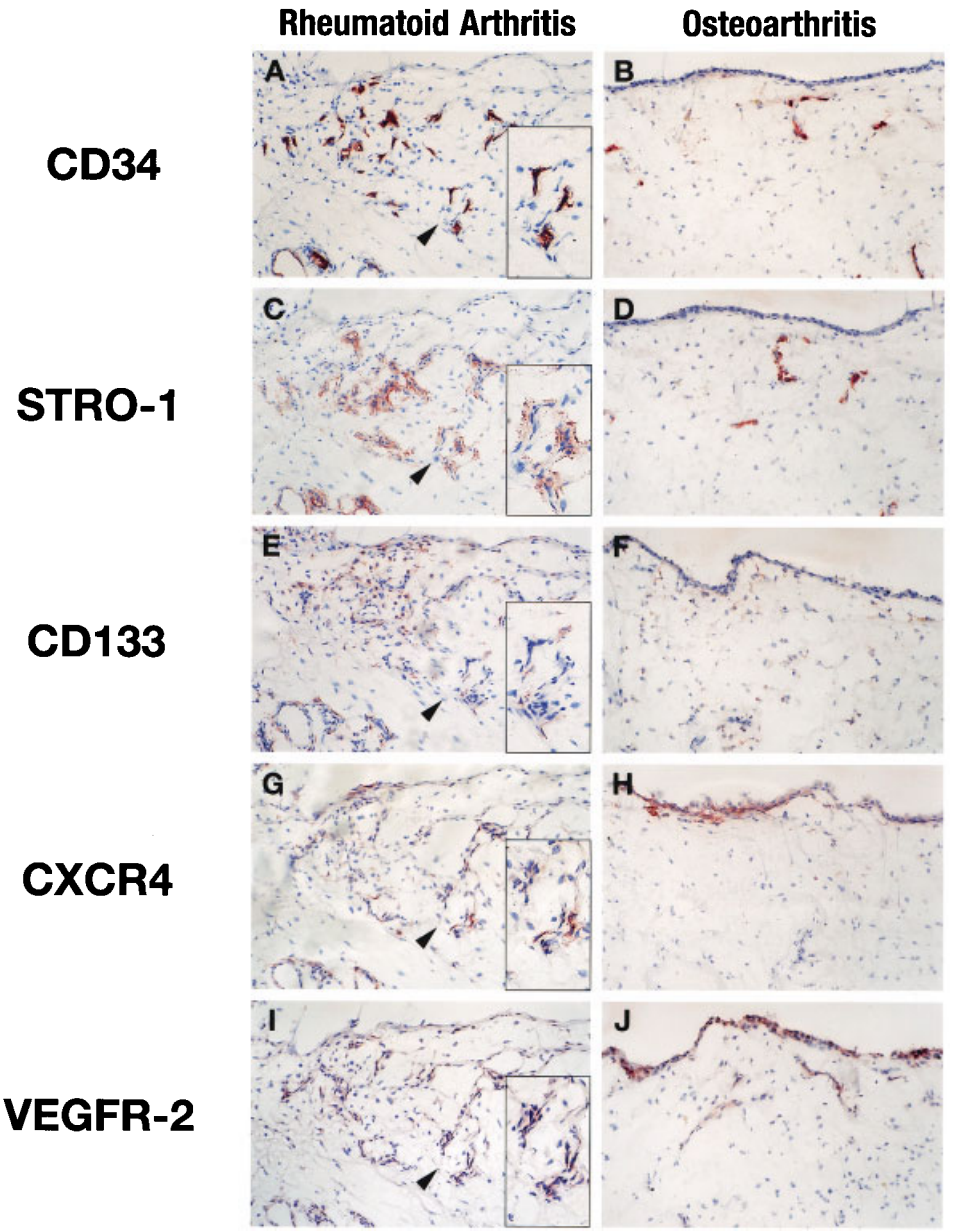

Figure 1. Expression of CD34, STRO-1, CD133, CXCR4, and vascular endothelial growth factor receptor 2 (VEGFR-2) on precursor cells in serial sections of synovial tissues obtained from 1 representative patient with rheumatoid arthritis (RA) and 1 representative patient with osteoarthritis (OA). Cryosections of synovial membranes from 1 RA patient (**A**, **C**, **E**, **G**, and **I**) and 1 OA patient (**B**, **D**, **F**, **H**, and **J**) were stained with the endothelial and stem cell marker CD34 (**A** and **B**), the mesenchymal progenitor cell marker STRO-1 (**C** and **D**), the stem cell and endothelial precursor cell marker CD133 (**E** and **F**), the marker CXCR4 (**G** and **H**), and the marker VEGFR-2 (**I** and **J**), and were counterstained with Mayer's hemalum. **Arrowheads** indicate an area of vasculogenesis that is shown at higher magnification in **insets** in **A**, **C**, **E**, **G**, and **I**, with CD34+ cells located on the inside (**inset** in **A**) surrounded by STRO-1+ cells (**inset** in **C**) and with CD133+ cells on the outside (**inset** in **E**). The CD34+ cells within this cluster expressed high levels of CXCR4 (**inset** in **G**), while VEGFR-2 was equally expressed on single CD34+ and single CD133+ cells (**inset** in **I**). The primary antibody was omitted for control purposes, and isotype controls were included in the protocol and showed no staining. (Original magnification × 150 in **A–J**; × 300 in **insets**.)

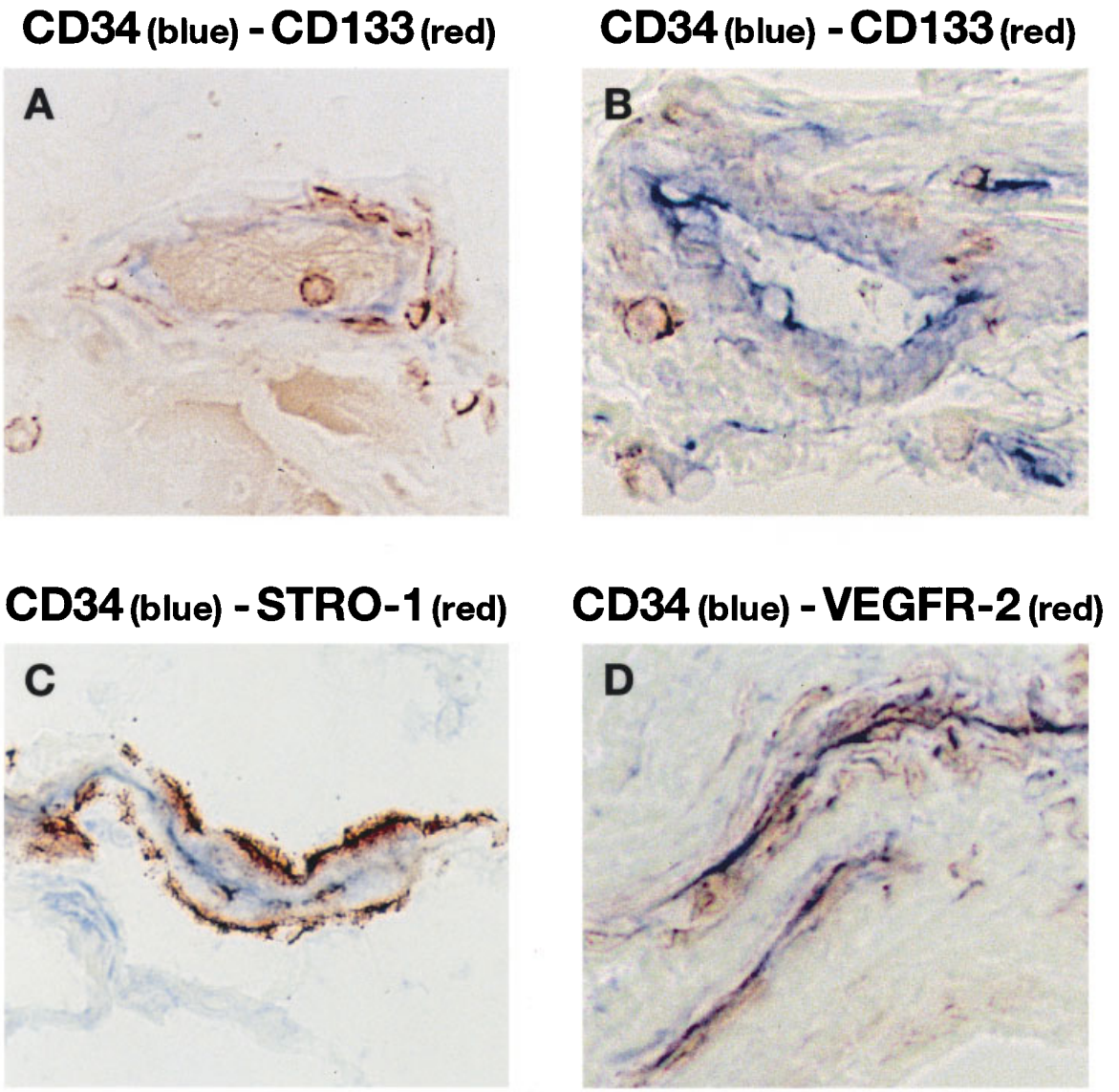

Figure 2. Two-color immunolabeling of endothelial precursor cells in a representative synovial tissue sample obtained from 1 RA patient. Synovial tissues from 1 RA patient were incubated with monoclonal antibodies to CD133 (**A** and **B**), STRO-1 (**C**), and VEGFR-2 (**D**) followed by biotinylated sheep $F(ab')_2$ anti-mouse Ig and a supersensitive streptavidin–horseradish peroxidase conjugate. Endogenous peroxidase was blocked as described in Materials and Methods, and antibody binding was visualized by exposure to 3-amino-9-ethylcarbazole. Tissue sections were counterstained with fluorescein isothiocyanate (FITC)–conjugated anti-CD34 (**A–D**) followed by a sequential incubation step with anti-FITC monoclonal antibody coupled to alkaline phosphatase and exposed to naphthol-AS-MX phosphate–Fast Blue BB. The primary antibody was omitted for control purposes, and isotype controls were included in the protocol and showed no staining. See Figure 1 for other definitions. (Original magnification × 400.)

synovial membranes of all RA and OA patients, although the expression varied among the patients (Figure 4). Of the 18 RA patients, only 14 showed CD133 mRNA expression, giving a PCR product with the predicted size of 449 bp. In all but 2 of these patients' tissues, transcripts for CXCR4 and VEGFR-2 were detected as well. Similarly, 11 of the 15 OA patients showed message for CD133, and those who showed a distinct band for CD133 also expressed message for CXCR4 and VEGFR-2. An identical parallel PCR for CXCR4 was performed using RNA samples that had not

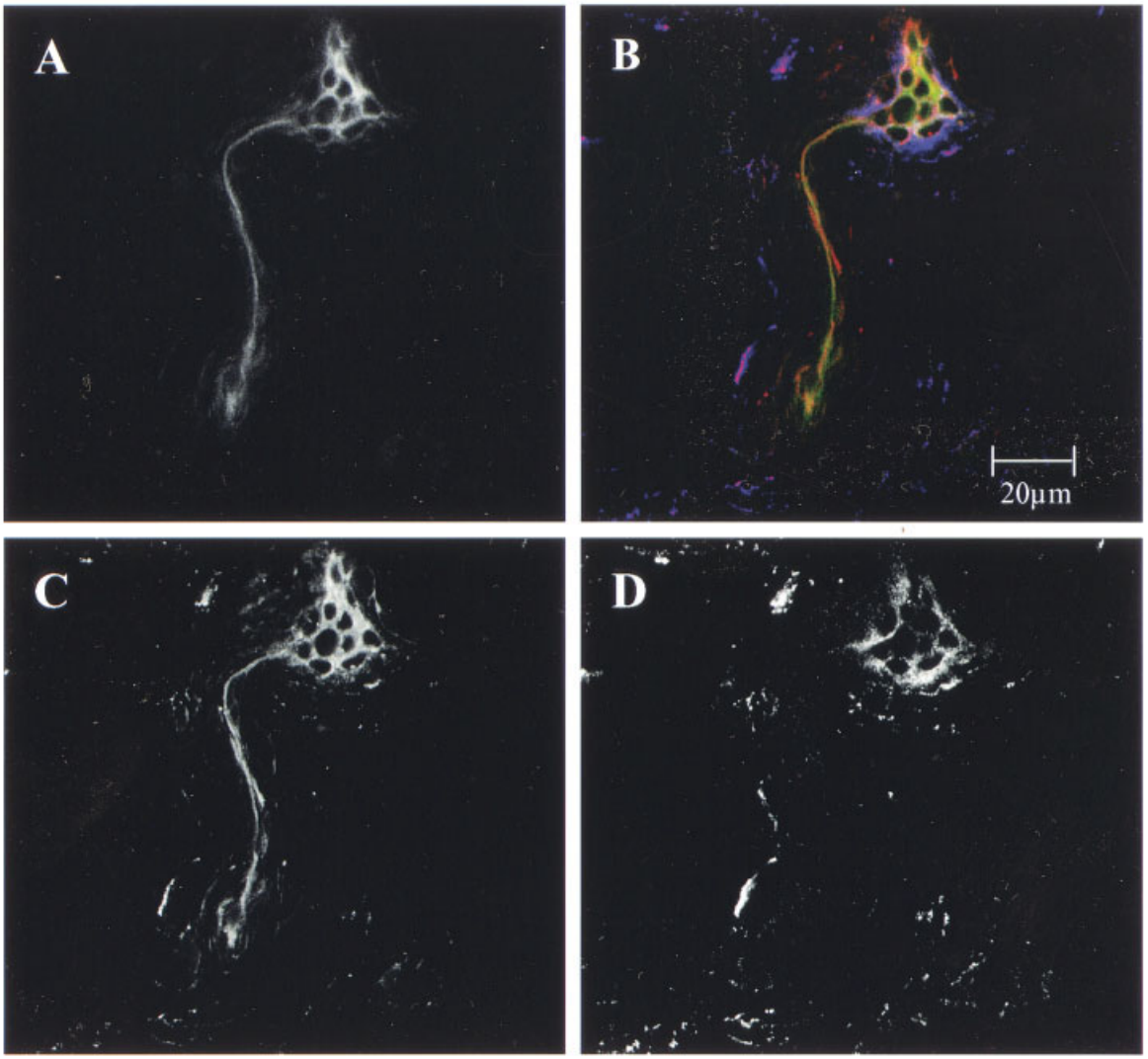

Figure 3. Three-color laser scans showing the expression of CD34, CD133, and STRO-1 on precursor cells in a synovial tissue sample from a representative patient with rheumatoid arthritis. Acetone-fixed synovial tissue sections (7 μm) were incubated with anti-CD133, subsequently blocked with 500 μg human IgG, and incubated with tetramethylrhodamine isothiocyanate–conjugated goat anti-mouse F(ab′)$_2$ antibody. Tissue samples were then blocked with mouse serum and incubated with anti–STRO-1 and fluorescein isothiocyanate–conjugated anti-CD34 overnight at 4°C. Staining of STRO-1 was revealed using biotin-conjugated goat anti-mouse IgM followed by streptavidin–Cy5. Serial dilutions of each primary and secondary antibody were tested to minimize nonspecific adsorption, assure separation of the fluorescent signals, and optimize fluorophore concentration to preclude self-quenching. Sections were analyzed with a confocal laser scanning microscope and a differential interference contrast oil immersion objective. Single scans showing precursor cells staining positive for CD34, CD133, and STRO-1 are shown in **A**, **C**, and **D**, respectively. Overlays of all 3 scans are shown in **B**. (Original magnification × 630.)

been reverse transcribed, because the gene for CXCR4 lacks intron sequences to control genomic contamination. No genomic contamination of the isolated RNA could be observed. Of the two isoforms of CD133 (i.e., AC133-1 and AC133-2) (24), only AC133-2 mRNA was found in the synovial tissue extracts of RA and OA patients (results not shown).

Endothelial precursor cells and activated T cells expressing CXCR4 can be attracted to synovial membrane via mechanisms involving stromal cell–derived factor 1 (SDF-1). To determine the quantity of the chemokine receptor CXCR4 message, a nonradioactive RNase protection assay was established in which 20 μg purified RNA extracted from the synovial tissue of RA and OA patients

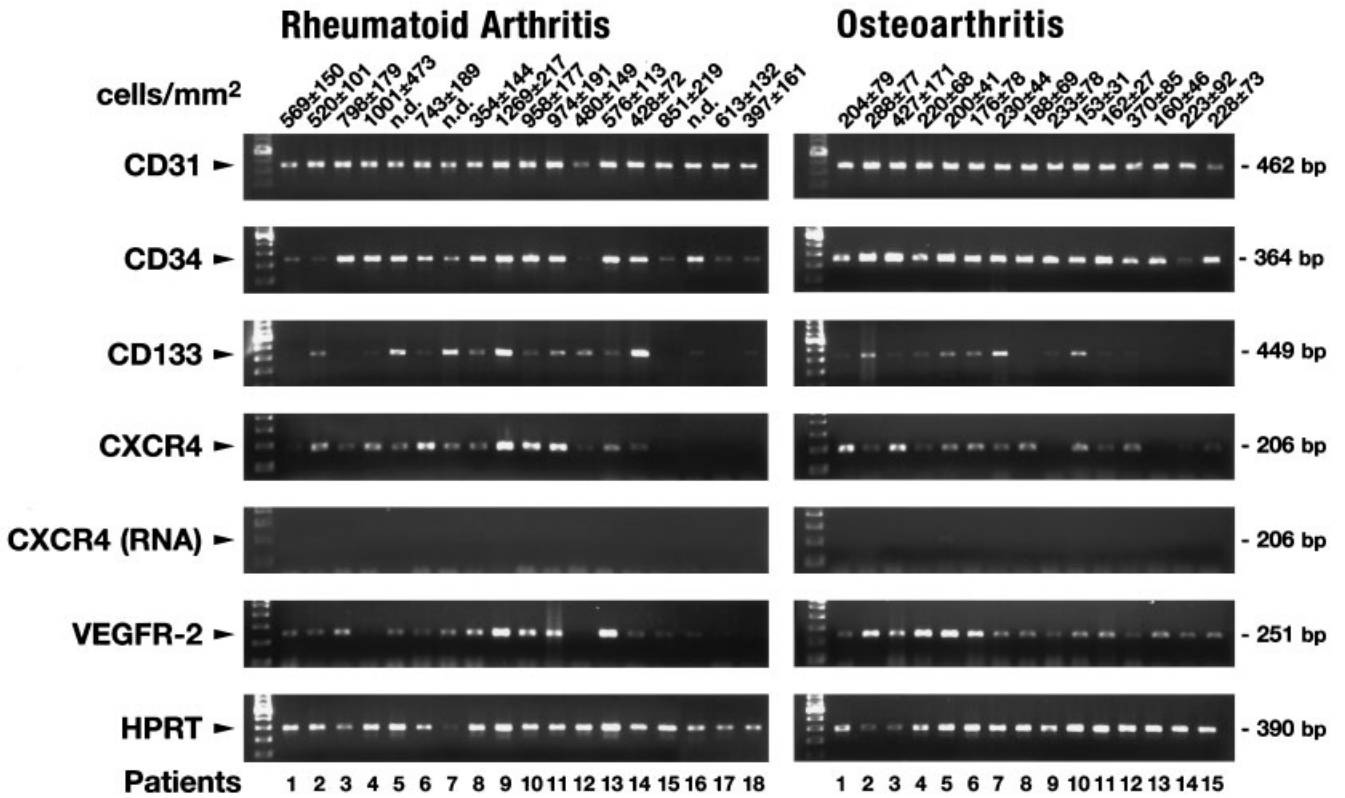

Figure 4. Total number of cells/mm^2 and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of CD31, CD34, CD133, CXCR4, and VEGFR-2 expression in synovial tissues of RA and OA patients. The total numbers of cells/mm^2 (mean ± SD) were determined by morphometric analysis in synovial membranes of 15 RA patients and 15 OA patients. RT-PCR analysis of CD31, CD34, CD133, CXCR4, and VEGFR-2 expression in synovial membranes of 18 RA patients and 15 OA patients shows bands with the predicted sizes of the PCR products. An identical parallel PCR for CXCR4 was performed using RNA samples that had not been reverse transcribed, because the gene for CXCR4 lacks intron sequences to control genomic contamination. The low-expressing housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) served as a control; a 100-bp DNA ladder size marker is shown in the far left lane of each panel. See Figure 1 for other definitions.

was used as starting material. Approximately 3–5 times more OA synovial tissue was needed to get the same amount of total RNA as that extracted from synovial membranes obtained from RA patients (data not shown). Of the 12 RA patients whom we investigated, samples from 11 showed message for CXCR4 and gave a band with the predicted size of 257 nucleotides; of the 10 OA patients, 8 showed message for CXCR4 (results not shown). Interestingly, the intensity of the 257-nucleotide band was slightly stronger in OA tissues, although more activated T cells were found in synovial tissues of RA patients (results not shown).

DISCUSSION

During inflammatory joint disease, the damaged articular cartilage and bone can be replaced by newly differentiated cells derived from precursor cells, or through local self repair by fully differentiated cells (3,4). To initiate tissue repair, a sufficient vascular supply is necessary, and blood vessels are mandatory for mesenchymal progenitor cells to migrate to the damaged joint. New vessels can be formed simultaneously by vasculogenesis, with circulating endothelial precursor cells migrating to the joint where they differentiate and proliferate, or by angiogenesis, which involves sprouting from preexisting vessels (12).

In the synovial tissue of 14 of 18 RA patients and in that of 11 of 15 OA patients, CD34+,CD31− precursor cells formed cell clusters with STRO-1+ cells and CD133+ precursor cells. Within these cell clusters, CD34+ precursor cells were in the center of the layer, surrounded by STRO-1+ cells and with CD133+ pre-

cursor cells on the outside. The majority of CD34+ cells in the cell clusters were STRO-1−, but they expressed CD133 with low intensity on their surfaces. CD133+ cells expressed CD34 with low intensity; STRO-1+ cells were CD34−, and some STRO-1+ cells expressed low levels of CD133.

In addition to these cell clusters, single CD133+ cells were found in the lumen of blood vessels and in perivascular areas within the synovial membranes of RA patients. It is fascinating to find and show a single circulating CD133+ cell in a vessel, since this cell population is extremely rare in the blood circulation. Morphometric analysis of RA synovium showed that higher numbers of CD133+ precursors and STRO-1+ cells were found, particularly in synovial membranes with lymphocyte ingress. Interestingly, in the synovial tissue of 11 of the 15 OA patients, precursor cells expressing CD34, CD133, or STRO-1 were found, and synovial tissue extracts showed message for CD34 and CD133. The intensities of the PCR bands corresponding to CD34 and CD133 mRNA expression were similar in RA and OA patients, since ~3–5 times more OA synovial tissue was needed to yield the same amount of total RNA as that isolated from RA synovium. Further, the total number of cells/mm^2 was 3–4 times greater in RA synovial membranes than in OA tissues. These results indicate that vasculogenesis is initiated in the synovial membranes of both RA and OA patients. In the synovial tissue of the remaining 4 OA patients, however, CD133+ cells and STRO-1+ mesenchymal progenitor cells were not detectable, and no CD133 message was observed.

The majority of CD34+ precursor cells within the cell layer expressed high levels of the chemokine receptor CXCR4, the receptor of SDF-1. The chemokine SDF-1 plays a central role in selective homing and recruitment of circulating stem cells and activated CD4+ T cells to the inflamed joint (25). SDF-1 can stimulate integrin-mediated arrest of CD34+,CXCR4+ stem cells on vascular endothelium under shear flow by increasing the adhesiveness of the integrins very late activation antigen 4 (VLA-4), VLA-5, and lymphocyte function–associated antigen 1 (26).

Furthermore, CD34+ progenitor cells and CD133+ endothelial precursor cells in the synovial tissue of RA and OA patients expressed high levels of VEGFR-2. VEGFR-2 mediates the major growth and permeability actions of VEGF, the most critical driver of vascular formation under various physiologic and pathologic conditions (15,16,27,28). VEGF consists of 4 major isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) assembled by alternative splicing. In the synovial tissue of RA and OA patients, $VEGF_{121}$ is constitutively expressed, while the expression of $VEGF_{165}$ is detected in only 41% of RA synovia and is not found in OA (28). Selective up-regulation of $VEGF_{165}$ and its signaling via VEGFR-2 can play an important role in synovial angiogenesis and vasculogenesis in RA. Herein we have shown that high levels of VEGFR-2 were expressed on the surface of two populations of precursor cells in synovial tissues of RA and OA patients, one population being CD34+ and the other being CD133+.

In contrast, the majority of STRO-1+ cells in the synovial tissue were VEGFR-2− but expressed α-SMA, a marker for subendothelial pericytes and smooth muscle cells, on their surfaces. This confirms the findings of previous studies in which STRO-1+ cells were found within the walls of the microvasculature of the human thymus (where a subset of them also expressed α-SMA) and in normal and hyperplastic pannus of RA patients (20,29–31). STRO-1+,α-SMA+ pericytes can be recruited from neighboring resident mesenchymal cells through replication, migration, and differentiation of other pericytes downstream of the growing vascular bud, or they can arise directly from endothelial cells or their progenitors. They are needed to coat the microvessels and stabilize them by preventing vessel pruning (18). Pericytes are one of the most elusive cell types in the body, and their significance as potential progenitor cells has been postulated repeatedly.

In general, STRO-1+ mesenchymal progenitors have an enormous plasticity; they can differentiate to chondroblasts, osteoblasts, adipocytes, and smooth muscle cells, and a fully differentiated chondrocyte can dedifferentiate in culture and then shift to an osteogenic phenotype (32–35). This highlights the nonirreversible nature of differentiation of cells from the mesenchymal cell lineage, otherwise seen as end points of various pathways. There is a physiologic need for plasticity of connective tissue cells in inflammation, namely, the need to adapt different tissues that reside next to one another during tissue repair.

In conclusion, we can say that endothelial progenitor cells are present in the synovial membranes of RA and OA patients and can form cell clusters to generate new vessels. De novo formation of blood vessels can help to prevent disease by allowing bone marrow–derived mesenchymal cells to migrate to the inflamed joint to induce tissue repair. New vessel growth, however, is not always desirable. Extensive vessel formation facilitates the proliferation of the pannus in RA by providing the growing cell mass with

sufficient blood supply. Leukocyte infiltration into synovial tissue depends on generation of new blood vessels, vessel formation is induced and modulated by cytokines and chemokines. Halting of blood vessel development could be an avenue of research potential new therapies.

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