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Focally Regulated Endothelial Proliferation and Cell Death in Human Synovium

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Angiogenesis and vascular insufficiency each may support the chronic synovial inflammation of rheumatoid arthritis. We have shown by quantitative immunohistochemistry and terminal uridyl deoxynucleotide nick end labeling that endothelial proliferation and cell death indices were each increased in synovia from patients with rheumatoid arthritis compared with osteoarthritic and noninflamed controls, whereas endothelial fractional areas did not differ significantly among disease groups. Markers of proliferation were associated with foci immunoreactive for vascular endothelial growth factor and integrin $\alpha_v\beta_3$, whereas cell death was observed in foci in which immunoreactivities for these factors were weak or absent. No association was found with thrombospondin immunoreactivity. The balance between angiogenesis and vascular regression in rheumatoid synovitis may be determined by the focal expression of angiogenic and endothelial survival factors. Increased endothelial cell turnover may contribute to microvascular dysfunction and thereby facilitate persistent synovitis. ***(Am J Pathol 1998, 152:691–702)***

Rheumatoid arthritis is a chronic inflammatory disease of synovial joints causing cartilage destruction, bone erosion, pain, and disability. The normal joint space is lined by macrophage- and fibroblast-like synoviocytes. This lining region is one or two cells deep and is highly vascular.[1] High synovial blood flow provides oxygen and nutrients to the synoviocytes and to the avascular articular cartilage. In rheumatoid arthritis, the synovial lining is hyperplastic and locally invasive, leading to bone and cartilage destruction.

Synovial hyperplasia in rheumatoid arthritis is caused by increased infiltration of inflammatory cells, including macrophages and lymphocytes, and also by proliferation of fibroblast-like cells.[2–4] The hyperplastic synovium contains numerous blood vessels. Vascularization of the expanding synovium may contribute to the pathogenesis of rheumatoid arthritis by providing oxygen and nutrients to support the high metabolic activity of synoviocytes, by permitting the migration of additional inflammatory cells into the joint, and by generating regulatory factors and matrix-degrading enzymes that exacerbate joint damage. Rheumatoid arthritis has accordingly been referred to as an angiogenic disease.[5] Endothelial cell proliferation is required for sustained angiogenesis.[6] Markers of proliferation, including [^{3}H]thymidine uptake, Ki-67 antigen, nuclear organizer regions, and proliferating cell nuclear antigen (PCNA) have been localized to endothelial cells in inflamed synovia.[2–4,7,8]

Despite this evidence of angiogenesis, synovium in rheumatoid arthritis is chronically hypoxic, and this hypoxia may contribute to synovial and cartilage damage.[9–11] Vascular density may be decreased in the synovial lining region in rheumatoid arthritis.[12,13] Synovial hypoxia and reduced vascular density indicate that angiogenesis does not keep pace with synovial metabolic demand and hyperplasia.

Rheumatoid arthritis is a persistent disease that afflicts patients for many decades. If angiogenesis is to be sustained, it must be balanced by vascular regression. In normal wound healing, angiogenesis is followed by vascular regression during the period when granulation tissue is reorganized to form scar tissue.[14] Vascular regression also occurs during normal embryogenesis and luteolysis.[15,16] Regression of blood vessels in these circumstances requires the death of endothelial cells, probably by the tightly regulated process of apoptosis as indicated by terminal uridyl deoxynucleotide nick-end labeling (TUNEL) of fragmented DNA. TUNEL-positive endothelial cells also have been observed in synovia from patients with rheumatoid arthritis.[17] This may indicate that vascular regression is a feature of this disease. The apparent paradox between increased endothelial proliferation and reduced vascular density persisting over many years may be explained by a concurrent increase in endothelial cell death in rheumatoid synovitis.

Vascular endothelial growth factor (VEGF) and thrombospondin, factors that respectively stimulate or inhibit angiogenesis, are each up-regulated in synovia from patients with rheumatoid arthritis.[8,18–21] VEGF and integrin $\alpha_v\beta_3$ act as survival factors for newly formed vessels, and

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Table 1. Clinical Details

Study/group	Age in years median (range)	Number (male)	Drug treatment (*n*)*
Endothelial PCNA index			
Control	59 (30–87)	4 (3)	None
Osteoarthritis	69 (57–80)	9 (2)	None
Rheumatoid arthritis	66 (43–88)	9 (2)	AZA (2), PA (1), G (1), Pred (1)
Endothelial TUNEL index			
Control	36 (19–51)	9 (6)	None
Osteoarthritis	59 (39–77)	8 (2)	None
Rheumatoid arthritis	62 (39–84)	10 (3)	MTX (2), AZA (1), Pred (2)
Integrin $\alpha_v\beta_3$, VEGF, and thrombospondin			
Control	40 (19–44)	6 (4)	None
Osteoarthritis	72 (52–84)	6 (4)	Pred (1)
Rheumatoid arthritis	71 (40–84)	11 (1)	MTX (1), SZ (1), G (1), Pred (3)

*Slow acting antirheumatic agents and glucocorticosteroids only are listed.
Abbreviations: AZA, azathioprine; G, gold; MTX, methotrexate; PA, D-penicillamine; Pred, prednisolone; SZ, sulphasalazine.

their expression or down-regulation may lead to persistence or regression of neovasculature, respectively.[22–24] We propose a model of chronic synovitis in which the balance between angiogenesis and vascular regression is determined by the focal expression of regulators of angiogenesis and endothelial cell survival. Increased vascular turnover, even in the absence of increased vascular density, may generate an immature, dysregulated vascular bed in which mismatch between perfusion and metabolic demand leads to synovial hypoxia and persistent inflammation.

Materials and Methods

Tissue Collection and Preparation

Human synovium was obtained during total joint replacement surgery from patients satisfying the American College of Rheumatism revised criteria for rheumatoid arthritis or osteoarthritis (Table 1).[25,26] Control synovia were obtained from radiologically normal knees during anterior cruciate ligament repair or carbon fiber resurfacing of articular cartilage for chondromalacia patellae and from postmortem subjects with no previous clinical history of arthritis. Normal human abdominal skin was obtained during abdominoplasty surgery, normal human kidney was obtained postmortem, and normal rat tissues from male Wistar rats following death by asphyxiation in carbon dioxide.

Specimens for endothelial PCNA index were fixed in formal saline before routine processing and embedding in paraffin wax. Four-μm sections were cut on a sledge microtome. For other studies, specimens were frozen to cork blocks in Tissue-Tek mounting medium (Miles, Elkhart, Indiana) by immersion in liquid nitrogen-cooled isopentane. Four-μm sections were then cut in a Bright OTF/AS-001 motorized cryostat (Bright, Huntingdon, U.K.). Sections were mounted on 3-aminopropyltriethoxysilane-pretreated slides before oven baking at 58°C (PCNA) or air drying at room temperature (TUNEL/Ki-67) for 1 hour.

Staining Procedures

For sequential staining of proliferation and endothelial markers, sections were immunostained first by the avidin-biotin complex (ABC)-peroxidase method for PCNA or Ki-67 antigen using monoclonal antibodies PC10 or Ki-67,[27,28] and then for endothelium by the ABC-alkaline phosphatase method using the monoclonal antibody QBEND10 directed against CD34,[29] or lectin immunohistochemistry using *Ulex europaeus* agglutinin-I.[30] Sections were stained for integrin $\alpha_v\beta_3$ using monoclonal antibodies LM609 and 23C6,[31,32] for VEGF using affinity purified rabbit polyclonal antibodies VEGF (A-20) and VEGF (147),[33] or for thrombospondin using mouse monoclonal antibodies P10 and 11.4.[34,35] Sections stained by TUNEL were sequentially stained for endothelium using lectin immunohistochemistry and visualized by immunofluorescence with Texas red-conjugated anti-goat IgG.

PCNA and Ki-67 Antigen Immunoreactivity

Paraffin-embedded sections (PCNA) were dewaxed, rehydrated, and washed in phosphate-buffered saline (PBS). Frozen sections (Ki-67) were immersed in ice-cold acetone for 10 minutes. Sections were washed twice in PBS and then incubated in primary antibody in bovine serum albumin (0.05% w/v) and normal horse serum (3% v/v) for 1 hour at room temperature. After two washes in PBS, sections were incubated with biotinylated horse anti-mouse IgG for 30 minutes, rinsed twice in PBS, and then incubated for 30 minutes with peroxidase-conjugated ABC. Sections were again washed in PBS and then developed with diaminobenzidine (DAB) using the glucose oxidase/nickel-enhanced method.[36] Positive and negative tissue controls included human skin, and, for PCNA, rat skin, small intestine, thymus, spleen, kidney, and liver were used.

TUNEL

Sections for TUNEL were processed according to the Boehringer Mannheim *in situ* cell death detection (fluo-

rescein) kit protocol. Briefly, sections were postfixed in 4% paraformaldehyde, pH 7.4, for 30 minutes and washed twice in PBS (10 minutes per wash). Sections were then incubated with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. Sections were again washed in PBS before incubation with the TUNEL reagents (terminal deoxynucleotide transferase and fluorescein-labeled nucleotide mixture) for 1 hour at 37°C. For positive experimental controls, sections of synovium were incubated for 10 minutes with DNase I (1 μg/ml in 30 mmol/L Tris, 140 mmol/L sodium cacodylate, 4 mmol/L magnesium chloride, 0.1 mmol/L dithiothreitol, pH 7.2) before TUNEL. For negative experimental controls, terminal deoxynucleotide transferase was omitted. Positive and negative tissue controls included rat skin, spleen, kidney, and liver.

Endothelial Markers

Sections stained for proliferation markers or by TUNEL were sequentially stained for endothelium. For PCNA indices, sections were incubated with QBEND10 for 1 hour, then stained by the Vector ABC-alkaline phosphatase method, and developed using Sigma *FAST*™ (4-chloro-2-methylbenzenediazonium/3-hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate (α-naphthol AS-MX) and Fast Red TR). Sections stained for Ki-67 antigen or by TUNEL were incubated overnight at 4°C with *Ulex europaeus* agglutinin-I in 10 mmol/L HEPES buffer (pH 8.5, containing 0.1 mmol/L $CaCl_2$ and 150 mmol/L NaCl), then for 2 hours with goat anti-lectin, and for 90 minutes either with biotinylated rabbit anti-goat IgG and stained using the Vector ABC-alkaline phosphatase method, developed with Sigma *FAST*™ (for Ki-67 index), or with Texas red-conjugated rabbit anti-goat IgG (for TUNEL index).

Nuclei were counterstained by a 10-minute incubation with the DNA ligand, 4,6-diamidino-2-phenylindole hydrochloride (DAPI; 0.001% in PBS containing 0.6% Tergitol Nonidet P-40).[37] After two washes in PBS, slides were mounted in glycerol (10% v/v in PBS).

The distributions of markers of proliferation and cell death were compared using consecutive frozen sections from five patients with rheumatoid arthritis by sequential staining for Ki-67 antigen or by TUNEL, followed by lectin immunohistochemistry with *Ulex europaeus* agglutinin-I.

Integrin $\alpha_v\beta_3$, VEGF and Thrombospondin Immunoreactivity

Regulators of angiogenesis were localized by the ABC-peroxidase method, developed in DAB using Sigma Fast DAB, counterstained with hematoxylin, dehydrated, and mounted in dibutylphthalate polystyrene xylene. An observer blinded to clinical details allocated dummy variables 0, 1, or 2 to cases according to whether absent, weak, or intense immunoreactivity was localized to each tissue structure. For sequential staining in synovia from six patients with rheumatoid arthritis, Ki-67 antigen was localized using the ABC-peroxidase method with glucose oxidase/nickel enhanced DAB development, followed by immunohistochemistry for angiogenesis regulators using the ABC-alkaline phosphatase method and developed with SigmaFAST™, and counterstained with DAPI. Sequential TUNEL-labeling and lectin immunohistochemistry were performed on sections consecutive to those used for sequential Ki-67- and angiogenesis regulator-immunohistochemistry.

All incubations were at room temperature and PBS washes were for 5 minutes unless otherwise stated. Hematoxylin and eosin stains were performed on sections consecutive to each series used for immunohistochemistry. Collagenous matrix was localized by polarizing light microscopy.

Proliferation and Cell Death Indices

An observer blinded to clinical details quantified those microscopic fields that contained the highest proportions of positively-labeled nuclei using a Zeiss IIe photomicroscope (Zeiss, Welwyn Garden City, U.K.) with a ×16 objective lens. Transmitted light and fluorescence images were each captured without moving the section using a 3-CCD camera (Sony, Japan) and analyzed using a Symphony image analysis system (Seescan, Cambridge, U.K.).

Color images were converted to monochrome images, a frame surrounding the area of interest was delineated, and the image was thresholded according to optical density to include all CD34- or lectin-positive blood vessels within 200 μm of the synovial surface. Nonvascular structures were excluded from the thresholded image automatically according to size criteria (area <50 μm^2) and interactively on morphological grounds. A mask was created of endothelium and applied over the image of PCNA-, Ki-67-, or TUNEL-positive nuclei. Positive nuclei falling within endothelium were counted, and the thresholded endothelial area and frame area were measured. The corresponding image of DAPI-positive nuclei was overlaid by the endothelial mask, and DAPI-positive endothelial cell nuclei were counted.

Endothelial indices were defined as the number of PCNA-, Ki-67-, or TUNEL-positive endothelial nuclei divided by the total number of endothelial nuclei. Endothelial fractional area was defined as the CD34- or lectin-positive area divided by the frame area.

Data Analysis

Data were analyzed using SPSS Advanced Statistics Software (Version 6.1; Chicago, IL). Endothelial PCNA and TUNEL indices and fractional areas were logit-transformed before statistical analysis. The optimum number of sections per case and number of fields per section were determined using 10 fields from each of 10 consecutive sections of synovium from a patient with rheumatoid arthritis. The cumulative number of fields that gave the lowest coefficient of variation was chosen as the number of fields per section to be quantified (PCNA index, 7; TUNEL index, 4). The number of sections per case was

selected to give an SE ± 25% of the mean (PCNA index, 4; TUNEL index, 3).

Endothelial PCNA and TUNEL indices and endothelial fractional areas were analyzed using multivariate analysis of variance. Homogeneity of variances were assessed by Cochran's test, and possible correlations between TUNEL index or PCNA index and endothelial fractional area were assessed by Bartlett's test of sphericity. Heterogeneity of dependent variables between disease groups was measured by Pillai's test. Effects of disease group on TUNEL or PCNA indices and endothelial fractional area were assessed by univariate F tests, and when these were significant, *P* values for differences between pairs of disease groups were derived using Wilks' test. Values are quoted as means (95% confidence interval). Graphical data were presented using GraphPad Prism, version 2 (GraphPad, San Diego, CA). Correlations among intensities of integrin $\alpha_v\beta_3$, VEGF, and thrombospondin-like immunoreactivities were assessed on ordinal data using Spearman correlation coefficients.

Materials

Monoclonal antibodies PC10 and Ki-67 were from Dako (High Wycombe, U.K.). Biotinylated horse anti-mouse, goat anti-rabbit, and rabbit anti-goat antibodies, normal horse, goat and rabbit sera, *Ulex europaeus* agglutinin-1, goat anti-lectin, Texas red-conjugated anti-goat IgG, and ABC were from Vector Laboratories (Peterborough, U.K.). Monoclonal antibodies QBEND10 and 23C6 were from Serotec (Oxford, U.K.), and LM609 and P10 were from Chemicon (Temecula, CA). Rabbit polyclonal antibodies VEGF(A-20) and VEGF(147) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody clone 11.4 and the *in situ* cell death detection kits (fluorescein) were from Boehringer Mannheim (East Sussex, U.K.). PBS concentrate was from Microgen (Cambridge, U.K.). DAPI and other reagents were from Sigma (Poole, U.K.).

Results

Synovial Morphology

Hematoxylin-counterstained sections revealed inflammatory cell infiltrates in synovia from patients with rheumatoid arthritis (Figure 1A) or osteoarthritis but not in controls. Acellular collagenous matrix was identified in 9 of 11 patients with rheumatoid arthritis, four of six patients with osteoarthritis, and one of six noninflamed controls. CD34 immunoreactivity and *Ulex europaeus* agglutinin-I binding were localized to vascular endothelium in all samples (Figure 1). However, in contrast to results on formalin-fixed tissues, QBEND10 gave only weak staining of endothelium in cryostat sections (not shown). Numerous microvessels were localized within 200 μm of the synovial surface in all disease groups (Figure 1, C to F).

Proliferation Markers and TUNEL

Immunoreactivities for PCNA and Ki-67 antigen had similar nuclear localizations in fixed and unfixed tissues respectively. In particular, staining of endothelial nuclei was observed using either proliferation marker (Figure 1, A, C, and G). PCNA- and Ki-67-immunoreactive endothelial cells most often were localized to regions of synovium containing nonendothelial cells immunoreactive for these proliferation markers in synovia from patients with rheumatoid arthritis (Figure 1, C and G). PCNA- and Ki-67-positive nuclei were localized to the basal epidermis in normal skin, and PCNA-positive nuclei were observed in small intestinal crypts and in germinal centers in thymus and spleen and only occasionally in normal kidney and liver (not shown).

TUNEL-positive endothelial nuclei were localized to regions of synovium containing nonendothelial cells labeled by TUNEL (Figure 1, B and E). Pretreatment with DNase I resulted in labeling of all nuclei, and omission of terminal deoxynucleotide transferase abolished labeling. TUNEL-positive nuclei were detected in normal rat skin and spleen, and only occasional nuclei were positive for TUNEL in normal rat liver and kidney (not shown).

Ki-67-immunoreactive and TUNEL-positive endothelial cells were each detected on consecutive sections of each synovial sample from five patients with rheumatoid arthritis. Markers of proliferation and of cell death each were localized to different foci in the synovium, and vessel profiles containing both Ki-67- and TUNEL-positive nuclei were not detected (Figure 1, G and H).

Proliferation and TUNEL Indices and Endothelial Fractional Areas

Endothelial PCNA index and TUNEL index were each increased in synovia from patients with rheumatoid arthritis compared with osteoarthritic and noninflamed controls, whereas endothelial fractional areas did not differ significantly between disease groups (Figure 2).

Variances within the dependent variable groups were similar (PCNA study, $C = 0.5$, $P = 0.3$; TUNEL study, $C = 0.4$, $P = 0.8$). There was no significant correlation between either of the endothelial indices and endothelial cell fractional area (PCNA study, Bartlett's determinant = 0.6, $P = 0.4$; TUNEL study, Bartlett's determinant = 1.3, $P = 0.3$). Significant heterogeneity was found between disease groups with respect to the dependent variables (PCNA study, $F = 4.1$, $P = 0.007$; TUNEL study, $F = 3.5$, $P = 0.01$).

Endothelial PCNA index differed significantly between disease groups ($F = 11.5$, $P = 0.001$). Endothelial PCNA index was higher in synovia from patients with rheumatoid arthritis (2.4%, 1.0 to 3.9%) than in controls (0.2%, 0.03 to 0.3%) ($t = 4.3$, $P = 0.0004$) and higher than in synovia from patients with osteoarthritis (0.6%, 0.1 to 1.0%)($t = 3.6$, $P = 0.002$) (Figure 2A). Mean endothelial Ki-67 index in synovia from five patients with rheumatoid arthritis was 2.4% (0.1 to 9.9%).

Endothelial TUNEL index differed significantly between disease groups ($F = 7.9$, $P = 0.002$). Endothelial TUNEL index was higher in synovia from patients with rheumatoid arthritis (8.1%, 3.3 to 19%) than in controls (0.7%, 0.2 to 1.5%) ($t = 4.0$, $P = 0.0006$) and higher than in synovia from patients with osteoarthritis (2.7%, 0.4 to 8.2%) ($t = 2.2$, $P = 0.04$) (Figure 2B). No significant difference was found between osteoarthritic and control synovia for either endothelial PCNA index ($t = 1.5$, $P = 0.2$) or endothelial TUNEL index ($t = 1.6$, $P = 0.1$) (Figure 2, A and B).

Endothelial fractional area did not differ significantly between disease groups in either part of the study (PCNA study, $F = 1.3$, $P = 0.3$; TUNEL study, $F = 0.4$, $P = 0.7$; Figure 2C).

Integrin $\alpha_v\beta_3$

Monoclonal antibodies LM609 and 23C6 gave similar distributions of immunoreactivity, and each was localized to lining cells and to vascular endothelium in human synovium. Data are presented for clone LM609. In noninflamed synovia integrin $\alpha_v\beta_3$ immunoreactivity was weak or absent (Figure 3A). In synovia from 10 of 11 patients with rheumatoid arthritis and from one of six with osteoarthritis, intense integrin $\alpha_v\beta_3$ immunoreactivity was localized to endothelial cells and smooth muscle cells of some blood vessels with adjacent vessels displaying weak or absent immunoreactivity (Figure 3, B and C). Ki-67 immunoreactive vascular endothelium was immunoreactive for integrin $\alpha_v\beta_3$ (Figure 4A). In contrast, staining of serial sections indicated that vessels with intense integrin $\alpha_v\beta_3$ immunoreactivity were not labeled by TUNEL (not shown). Ki-67-immunoreactive nonvascular cells were localized to lining regions displaying intense integrin $\alpha_v\beta_3$ immunoreactivity (Figure 4B). Integrin $\alpha_v\beta_3$ immunoreactivity was localized to glomeruli in normal human kidney, whereas staining was weak in normal human skin (not shown).

Vascular Endothelial Growth Factor

Polyclonal antibodies VEGF(A20) and VEGF(147) revealed similar distributions of VEGF immunoreactivity, and data are presented for VEGF(A20). Intense VEGF immunoreactivity was localized to vascular endothelium in 6 of 11 patients with rheumatoid arthritis (Figure 3D) but in none of 6 patients with osteoarthritis nor of 6 noninflamed controls (not shown). Among cases, the intensity of endothelial VEGF immunoreactivity correlated with the intensity of endothelial integrin $\alpha_v\beta_3$ immunoreactivity ($r = 0.80$, $P < 0.001$). Within cases, endothelial VEGF immunoreactivity colocalized with integrin $\alpha_v\beta_3$ immunoreactivity (Figure 3, C and D). Intense VEGF immunoreactivity was localized to lining cells in 7 of 11 patients with rheumatoid arthritis (Figure 4C) and three of six patients with osteoarthritis but not in noninflamed controls. Regions with intense VEGF immunoreactivity colocalized with Ki-67-immunoreactive, TUNEL-negative nuclei (Figure 4, C and D). VEGF immunoreactivity was localized to keratinocytes in normal human skin, whereas dermal vessels displayed weak or absent immunoreactivity (not shown).

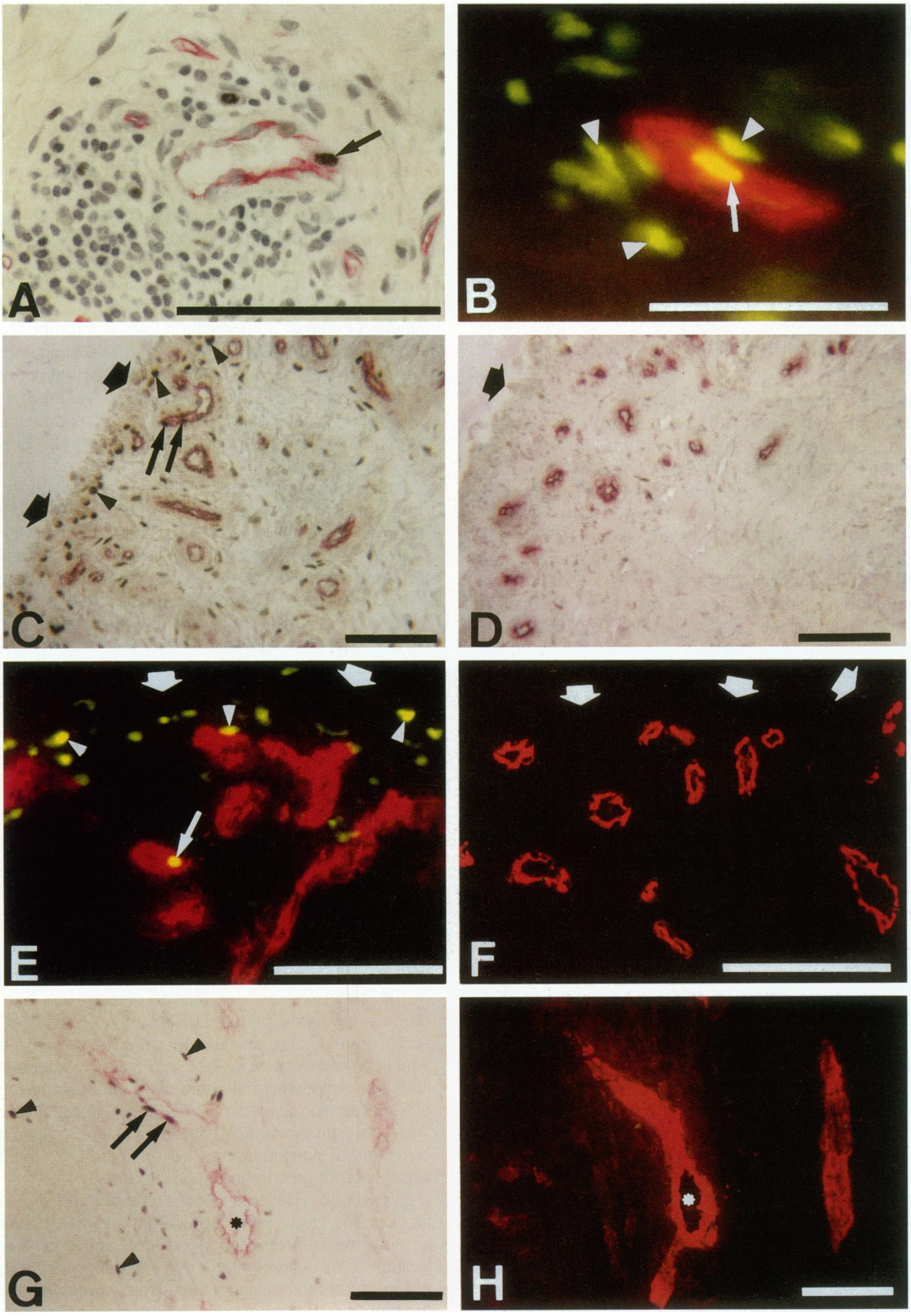
Thrombospondin

Monoclonal antibodies P10 and 11.4 revealed similar distributions of thrombospondin immunoreactivity, and data are presented for clone P10. Thrombospondin immunoreactivity was localized to acellular collagenous matrix in all disease groups (Figures 3E and 4E). Intense thrombospondin immunoreactivity was localized to the endothelium and smooth muscle of occasional blood vessels in 4 of 11 patients with rheumatoid arthritis (Figure 3F) but not in patients with osteoarthritis nor in noninflamed controls. Endothelial nuclei in thrombospondin immunoreactive vessels were neither Ki-67 immunoreactive nor TUNEL-positive. Thrombospondin immunoreactivity was localized to the synovial lining regions in 6 of 11 patients with rheumatoid arthritis, two of six patients with osteoarthritis, but not in noninflamed controls. Between cases, the intensity of vascular thrombospondin immunoreactivity correlated with the intensity of endothelial integrin $\alpha_v\beta_3$ immunoreactivity ($r = 0.44$, $P = 0.03$) and with the intensity of endothelial VEGF immunoreactivity ($r = 0.47$, $P = 0.02$). However, within cases, the distribution of thrombospondin immunoreactivity was distinct from that of VEGF (Figure 4, C and E), and integrin $\alpha_v\beta_3$ immunoreactivity, although some lining regions displayed immunoreactivity for all three markers (not shown). Thrombospondin immunoreactivity was localized to basement membranes around sebaceous glands in normal human skin (not shown).

Discussion

We have shown that markers of endothelial proliferation and markers of endothelial cell death are simultaneously present in discrete foci within synovium. Furthermore, the endothelial proliferation and cell death indices were higher in synovia from patients with rheumatoid arthritis than from those with osteoarthritis and noninflamed controls. This increase in both indices suggests that endothelial cell turnover is increased in rheumatoid synovitis. A balance between endothelial proliferation and cell death is indicated by the similarities in endothelial fractional area among disease groups. Vessels exhibiting intense immunoreactivity for integrin $\alpha_v\beta_3$ contained proliferating endothelial cells and were localized to regions of synovium populated by VEGF immunoreactive cells. The focal nature of endothelial cell proliferation and death in inflamed synovium may be explained by the focal expression of growth and survival factors.

Synovia from patients with rheumatoid arthritis used in the current study were obtained at joint replacement surgery and represented persistent, active disease as indicated by inflammatory cell infiltration on a background of chronic tissue repair, which was indicated by the high prevalence of collagenous matrix. The localizations in normal control tissues and to nonvascular cells in human synovium were as previously described for prolif-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eration markers,[2,4] TUNEL-labeling,[17,38–40] and immunoreactivities for integrin $\alpha_v\beta_3$,[32,41,42] VEGF,[8,18,19,43] and thrombospondin.[20,21,44]

Endothelial Proliferation Indices

PCNA and Ki-67 antigen are up-regulated in proliferating nuclei, and indices using these markers correlate with [^{3}H]thymidine uptake.[45,46] Nuclei immunoreactive for PCNA and Ki-67 antigen each had similar distributions, and similar proportions of endothelial nuclei were positive for each proliferation marker in synovia from patients with rheumatoid arthritis. Our data extend previous observations of endothelial proliferation in synovia from patients with rheumatoid arthritis.[3,7,8] Endothelial proliferation is a prerequisite for sustained angiogenesis,[6] and endothelial proliferation indices are increased during angiogenesis both in human tumors and in animal models.[47,48] Increased endothelial proliferation indices in synovia from patients with rheumatoid arthritis, compared with those with osteoarthritis and noninflamed controls, suggests increased angiogenesis in rheumatoid synovitis.

Endothelial TUNEL Index

The TUNEL method detects free 3′hydroxyl terminals of DNA and is therefore a marker of the DNA fragmentation that accompanies cell death. Labeling of 3′hydroxyl terminals is relatively specific for apoptosis but can occur in necrosis.[49–51] We have localized TUNEL-labeled endothelial nuclei in human synovium. Endothelial TUNEL-labeling has also been observed in regressing vessels during embryogenesis, wound healing, and luteolysis.[14–16] Increased endothelial TUNEL indices in synovia from patients with rheumatoid arthritis compared with those from patients with osteoarthritis and from noninflamed controls suggests increased vascular regression in rheumatoid synovitis.

Endothelial Fractional Area

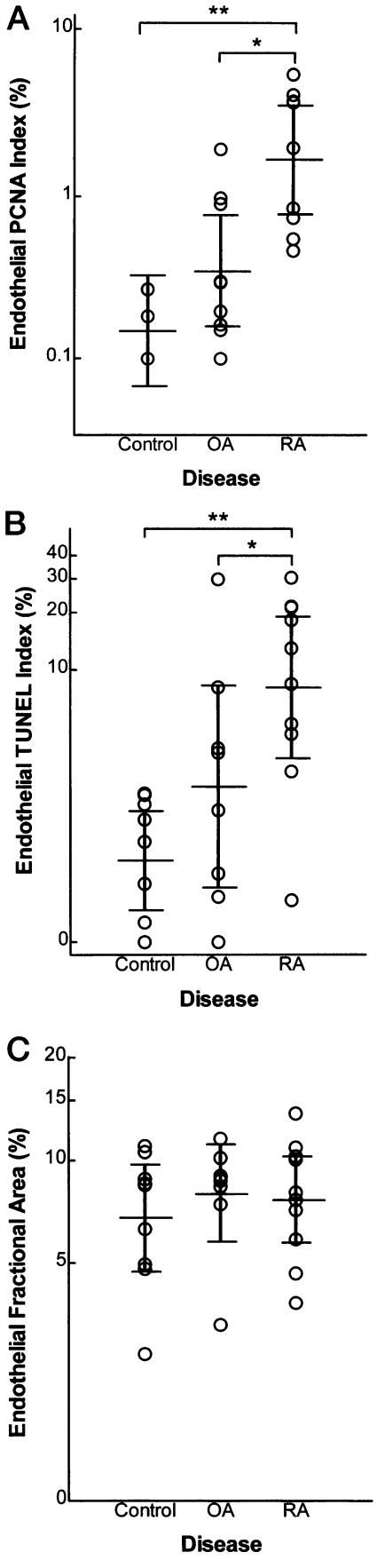
Endothelial fractional area is a measure of vascular density. Increased vascular density reflects angiogenesis in human tumors and correlates with increased tumor growth and metastatic rates.[47,52,53] The normal synovial lining region is highly vascular, and additional increases in vascular density have not consistently been observed in rheumatoid synovitis.[12,13,54] There is a redistribution of vessels in rheumatoid arthritis with a decreased vascular density in the superficial layers of synovium within 60 μm of the joint cavity and an increased vascular density in deeper synovium. Our data support the view that this redistribution of blood vessels results in no overall increase in vascular density in the superficial 200 μm of synovia from patients with rheumatoid arthritis. Previous studies have demonstrated increases in stromal cell proliferation or in stromal cell death in rheumatoid synovitis.[4,8,17,39] This may indicate that stromal hyperplasia represents a dynamic equilibrium between growth and regression. Our findings lead us to suggest that vascularization of the hyperplastic synovium depends also on an equilibrium between angiogenesis and endothelial cell death.

Ki-67 antigen- and TUNEL-positive nuclei each were observed in consecutive sections of synovia from patients with rheumatoid arthritis. Markers of proliferation and cell death were localized to discrete foci within the synovium rather than there being groups of cells containing both markers. In particular, individual blood vessels did not contain endothelia with both Ki-67 immunoreactive nuclei and TUNEL-positive nuclei. Endothelial proliferation and cell death therefore appear to occur concurrently but in distinct regions of synovium. Discrete foci of angiogenesis and of vascular regression may contribute to the endothelial heterogeneity observed in previous studies on human synovium. In particular, expression of endothelial antigens such as angiotensin converting enzyme and of vasoregulatory systems, such as substance P-containing sensory nerves and their receptors, increase during vascular maturation.[48,55] Vessels in rheumatoid synovium may represent a cross section of all stages of vascular maturity, some being newly-formed, others undergoing vascular regression.

Regulators of Vascular Turnover

The hyperplastic synovial stroma may regulate endothelial proliferation and survival. VEGF can stimulate angiogenesis, and both VEGF and integrin $\alpha_v\beta_3$ facilitate the survival of newly formed vessels.[22–24] Thrombospondin can inhibit angiogenesis *in vivo*.[56] This study and previous reports demonstrate that each of these factors is up-regulated in the synovia of patients with rheumatoid arthritis.[18,20,57] Our findings suggest that angiogenic and antiangiogenic factors are concurrently up-regulated in distinct foci of the inflamed synovium.

Figure 1. Markers of endothelial proliferation and cell death in human synovium. **A:** Synovium from a patient with rheumatoid arthritis stained for PCNA (**black**) and for the endothelial marker CD34 (**red**) showing PCNA-positive endothelial nucleus (**arrow**) in a venule surrounded by a focal lymphoid aggregate. Hematoxylin counterstain. **B:** Synovium from a patient with rheumatoid arthritis labeled by TUNEL (**green**) and by *Ulex europaeus* agglutinin-I binding for endothelium (**red**). An endothelial nucleus is TUNEL-positive (**arrow**) as are some nonendothelial nuclei (**arrowheads**). **C** and **D:** Synovia from a patient with rheumatoid arthritis (**C**) and from a nonarthritic postmortem control (**D**) stained for PCNA (**black**) and for CD34 (**red**). Both display a high density of microvessels near the synovial surface (**broad arrows**), whereas only the synovium from the patient with rheumatoid arthritis contains endothelial nuclei (**fine arrows**) and nonendothelial nuclei (**arrowheads**) immunoreactive for PCNA. **E** and **F:** Synovia from a patient with rheumatoid arthritis (**E**) and from a patient with chondromalacia patellae (**F**) labeled by TUNEL (**green**) and by *Ulex europaeus* agglutinin-I binding (**red**). Both display a high density of microvessels near the synovial surface (**broad arrows**), whereas only the synovium from the patient with rheumatoid arthritis displays a TUNEL-positive endothelial nucleus (**fine arrow**) and several TUNEL-positive nonendothelial nuclei (**arrowheads**). **G:** Synovium from a patient with rheumatoid arthritis stained for Ki-67 antigen (**black**) and by *Ulex europaeus* agglutinin-I binding (**red**). Ki-67 immunoreactive endothelial nuclei (**arrows**) and nonendothelial nuclei (**arrowheads**) occur in the same region. **H:** Consecutive section to that shown in **G** labeled by TUNEL (**green**) and by *Ulex europaeus* agglutinin-I binding (**red**). TUNEL-positive nuclei were not identified in this region, but were present elsewhere in this sample of synovium. * Corresponding vascular lumen in **G** and **H**. Scale bars, 100 μm (**A**, and **C** to **H**); 50 μm (**B**).



Intense integrin $\alpha_v\beta_3$ immunoreactivity was localized to proliferating endothelium in inflamed synovium. Integrin β_3 subunit immunoreactivity is increased on vascular endothelium and smooth muscle in synovia from patients with rheumatoid arthritis and correlates with inflammatory scores.[57] Integrin β_3 may form heterodimers with either α_v or α_{IIb} subunits. Monoclonal antibody 23C6 precipitates the integrin $\alpha_v\beta_3$ heterodimer from human osteoclasts, and LM609 recognizes an epitope that depends on both subunits of the integrin $\alpha_v\beta_3$ molecule.[31,32] Our data therefore support the view that up-regulated integrin β_3 subunit corresponds to an increase in integrin $\alpha_v\beta_3$ heterodimers on vascular endothelium in synovia from patients with rheumatoid arthritis. Vascular integrin $\alpha_v\beta_3$ is also up-regulated in healing skin wounds and in the chick chorioallantoic membrane model of angiogenesis.[41] Colocalization of proliferation markers and integrin $\alpha_v\beta_3$ to vessels, therefore, may indicate foci of angiogenesis in synovia from patients with rheumatoid arthritis. LM609 and cyclic peptide antagonists of α_v integrins each can inhibit angiogenesis by enhancing apoptosis in those endothelial cells that have recently entered the cell cycle.[23,41] Integrin $\alpha_v\beta_3$ immunoreactive and proliferating endothelial cells did not label by TUNEL in synovia from patients with rheumatoid arthritis. Up-regulation of integrin $\alpha_v\beta_3$ may contribute to the survival of synovial neovasculature, and down-regulation may subsequently permit vascular regression.

Our findings support previous reports that VEGF is up-regulated in synovia from patients with rheumatoid arthritis.[8,18,19] Furthermore, we found that VEGF is present in proliferative regions of inflamed synovium, regions in which angiogenesis is increased. This is consistent with a role for VEGF in stimulating new blood vessel formation in rheumatoid synovitis. VEGF also is a survival factor for vascular endothelial cells.[24] The absence of TUNEL-labeled endothelial cells in VEGF immunoreactive regions, therefore, may indicate a role for VEGF in inhibiting vascular regression in inflamed synovia.

Thrombospondin immunoreactivity was predominantly localized to acellular collagenous matrix in the deeper synovial regions in all disease groups. A similar localization of thrombospondin to extracellular matrix has also been described in other normal tissues and during inflammation and neoplasia.[35,44,58,59] In inflamed synovium, thrombospondin was also localized to the lining region and to some blood vessels as previously described.[20,21] Regions immunoreactive for throm-

Figure 2. Endothelial indices and vascular density within 200 μm of the synovial cavity in human synovium. **A:** Percentage of endothelial nuclei positive for proliferating cell nuclear antigen (endothelial PCNA index) in synovia from patients with osteoarthritis (**OA**) or rheumatoid arthritis (**RA**) compared with nonarthritic synovium (**control**). Endothelial PCNA index was higher in RA than in OA (* $t = 3.6$, $P = 0.002$) and higher in RA than in the control group (** $t = 4.3$, $P = 0.0004$). **B:** Percentage of endothelial nuclei positive for TUNEL (endothelial TUNEL index). Endothelial TUNEL index was higher in RA than in OA (* $t = 2.2$, $P = 0.04$) and higher in RA than in the control group (** $t = 4.0$, $P = 0.0006$). **C:** Percentage of synovial section within 200 μm of the synovial cavity occupied by *Ulex europaeus* agglutinin-I-positive endothelium (endothelial fractional area). No significant differences were found between RA, OA, and control groups. Data were derived from microscopic fields analyzed in **B**. Graphs show mean values for each patient (○), group means, and 95% confidence intervals.

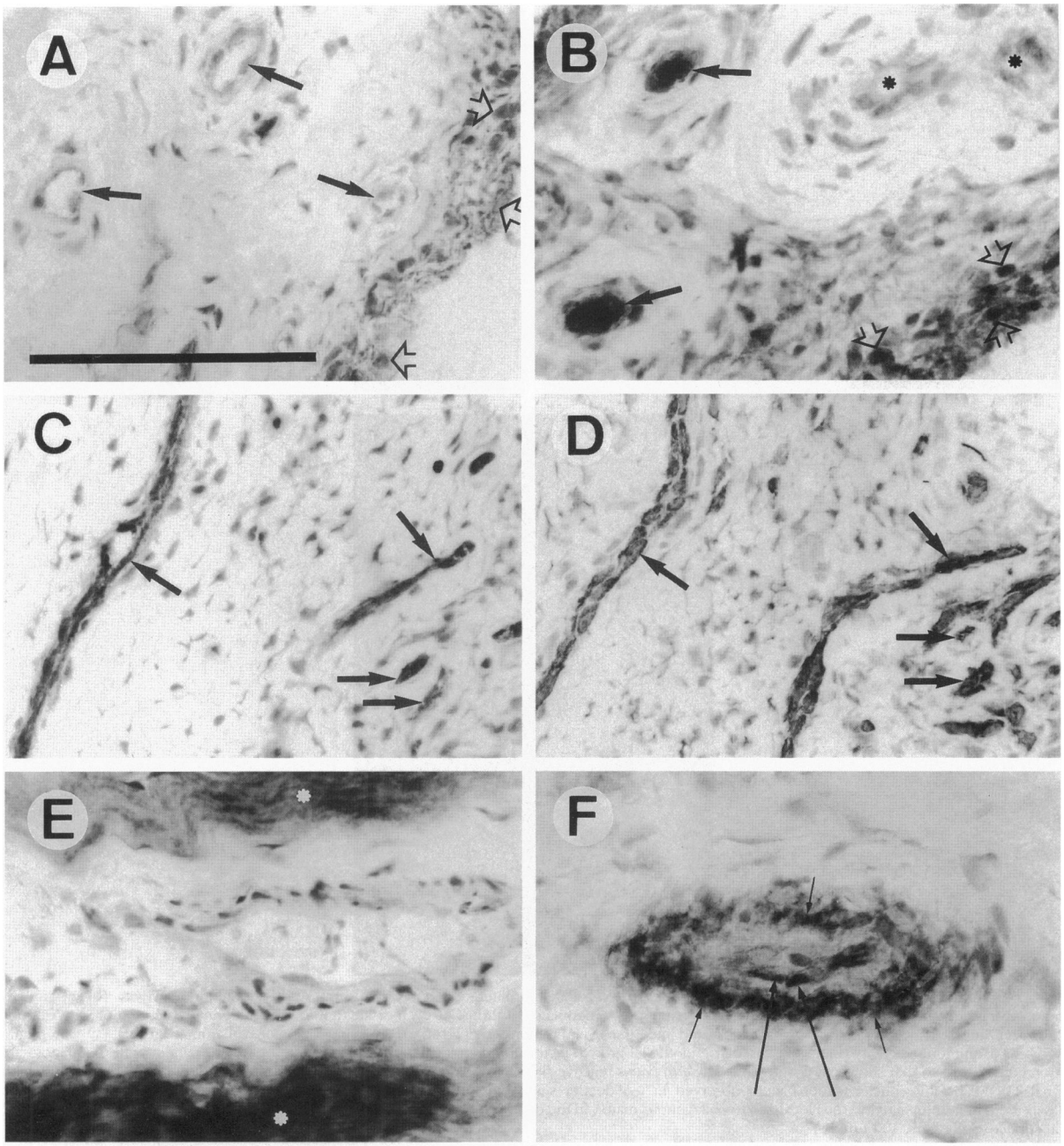


Figure 3. Regulators of angiogenesis in human synovium. **A:** Integrin $\alpha_v\beta_3$ immunoreactivity in noninflamed synovium from a patient with chondromalacia patellae showing absence of vascular staining (**solid arrows**) and weak staining of lining cells (**open arrows**). **B:** Integrin $\alpha_v\beta_3$ immunoreactivity in synovium from a patient with rheumatoid arthritis showing intense staining of some vessels (**solid arrows**) and lining cells (**open arrows**) and weak or absent staining in adjacent vessels (**asterisk**). **C:** Integrin $\alpha_v\beta_3$ immunoreactivity localized to vascular endothelium (**solid arrows**) in synovium from a patient with rheumatoid arthritis. **D:** Vascular endothelial growth factor immunoreactivity in a section of synovium consecutive to that shown in **C. Arrows** indicate colocalization with integrin $\alpha_v\beta_3$ immunoreactivity. **E:** Thrombospondin immunoreactivity localized to collagenous matrix (**asterisks**) in synovium from a patient with rheumatoid arthritis, surrounding a vessel in which immunoreactivity is absent. **F:** Thrombospondin immunoreactivity localized to vascular endothelium (**long arrows**) and smooth muscle (**short arrows**) in synovium from a patient with rheumatoid arthritis. Sections stained by the avidin-biotin peroxidase technique, developed in diaminobenzidine, and counterstained with hematoxylin. Scale bar, 100 μm.

bospondin did not consistently contain either proliferating or dying endothelial cells. Thrombospondin is a multifunctional protein that, in its soluble form, can inhibit angiogenesis but when bound to matrix proteins, can enhance the formation of new blood vessels.[56,60] Furthermore, several disulfide bonded isoforms of thrombospondin exist with differing biological activities and distributions.[20,21] It is possible that vascular turnover is regulated by isoforms of thrombospondin that are not specifically recognized by monoclonal antibodies P10 and 11.4 and modulated by additional factors in the synovial environment.

Focal and concurrent angiogenesis and vascular regression in chronically inflamed synovium may be explained by focal expression of angiogenic and survival factors such as integrin $\alpha_v\beta_3$ and VEGF. Other regulatory

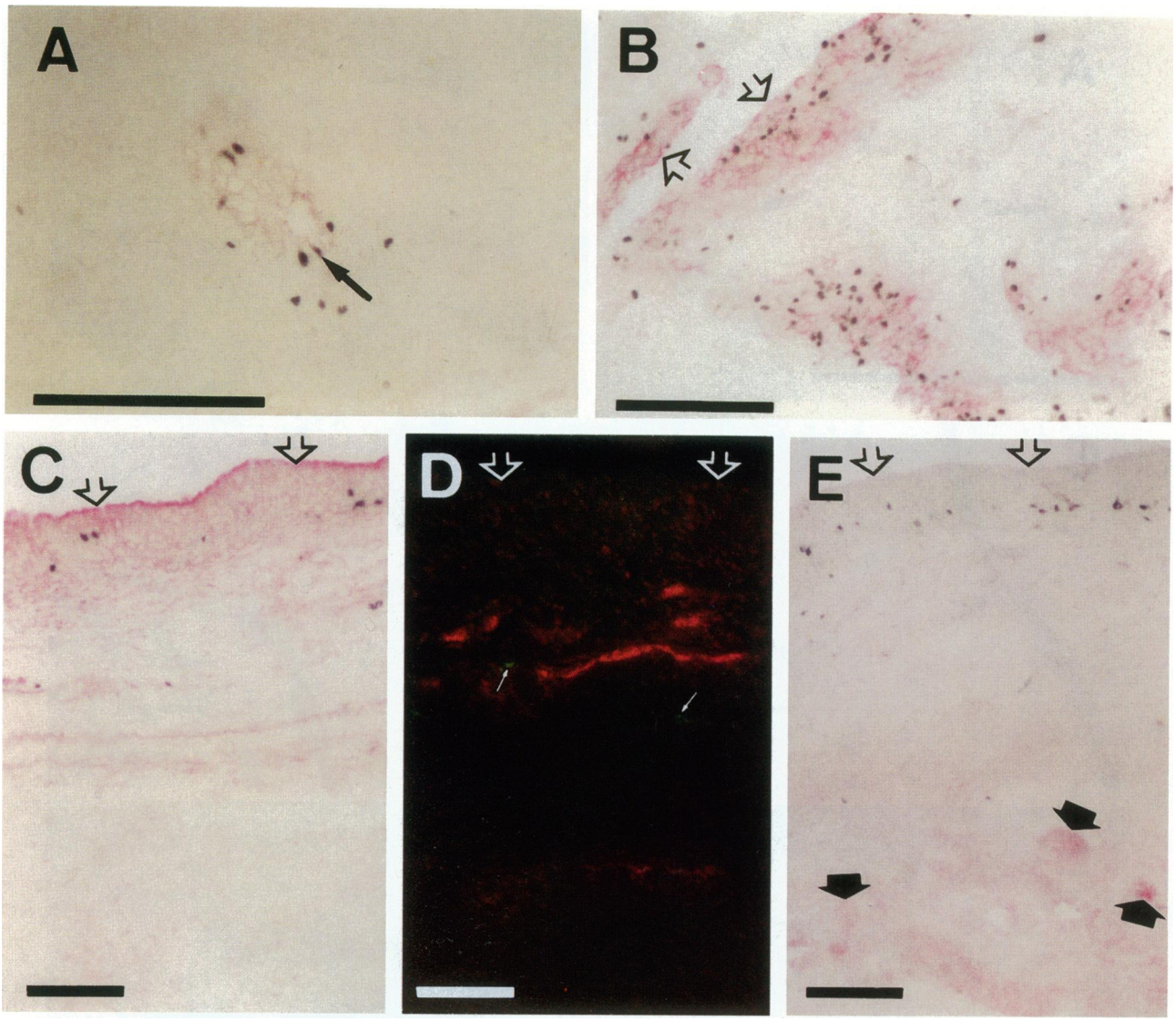


Figure 4. Colocalization of regulators of angiogenesis with markers of cell proliferation and cell death. **A:** Ki-67 immunoreactivity (**black**) in a vessel immunoreactive for integrin $\alpha_v\beta_3$ (**red**) in synovium from a patient with rheumatoid arthritis. **Arrow** indicates a Ki-67 and integrin $\alpha_v\beta_3$ immunoreactive endothelial cell. **B:** Colocalization of Ki-67 immunoreactivity (**black**) and integrin $\alpha_v\beta_3$ immunoreactivity (**red**) in the lining region of synovium from a patient with rheumatoid arthritis. **C:** Ki-67 immunoreactivity (**black**) in vascular endothelial growth factor immunoreactive (**red**) synovial lining from a patient with rheumatoid arthritis. **D:** Section consecutive to that shown in **C** showing *Ulex europaeus* reactive blood vessels (**red**). Nuclei in the lining region did not label by TUNEL, although occasional TUNEL positive nuclei (**green**) were observed in the deeper synovium (**small arrows**). **E:** Section consecutive to that shown in **D** showing thrombospondin immunoreactivity (**red**) localized to collagenous matrix in the deep synovium (**broad arrows**) but not to the lining region. **Open arrows** indicate synovial surface. Scale bars, 100 μm.

factors may also contribute to synovial vascular turnover. Thymidine phosphorylase and basic fibroblast growth factor can stimulate angiogenesis, and tumor necrosis factor-α increases endothelial cell death by apoptosis *in vitro*.[61] These factors are also up-regulated in synovia from patients with rheumatoid arthritis.[62–64] Inflamed synovium appears to be functionally compartmentalized with opposing processes of angiogenesis and vascular regression being concurrently and differentially regulated in separate foci.

The hyperplastic synovium has been likened to a neoplastic tumor and may contribute to joint damage in rheumatoid synovitis.[65] We have found that endothelial cells positive for proliferation markers were localized to regions of synovium containing proliferating nonendothelial cells. Angiogenesis may potentiate tissue hyperplasia by enhancing inflammatory cell influx and by the generation by endothelial cells of mitogenic factors such as basic fibroblast growth factor and angiotensin II.[64,66] The angiogenesis inhibitor TNP-470 suppresses synovitis and joint damage in rodent models of arthritis, indicating that neovascularization may exacerbate at least the early phases of chronic inflammation.[67,68]

In contrast to neoplastic tumors in which increased vascular density is associated with poor prognosis, decreased vascular density is associated with human arthritis. The synovium is chronically hypoxic in rheumatoid arthritis with biochemical evidence of anaerobic metabolism suggesting that blood flow is insufficient to meet the high metabolic demands of inflamed synovial tissue.[11,69]

Hypoxia may stimulate angiogenesis in rheumatoid synovitis through up-regulation of VEGF.[70] Reciprocally, synovial hypoxia may be exacerbated by focal vascular regression. Increased vascular turnover may result in immature vasoregulatory systems and additional mismatch between perfusion and metabolic demand. Hypoxia and acidosis can aggravate inflammation, pain, and tissue damage and may therefore promote the persistence of arthritis.[69]

The effects of angiogenesis inhibitors in rheumatoid synovitis may differ from those in short term animal models of arthritis or in neoplasia. Reducing vascular turnover may be a more important therapeutic aim than reducing vascular density. Understanding the mechanisms underlying increased endothelial cell turnover in rheumatoid arthritis may eventually lead to novel therapeutic strategies that alter the balance between angiogenesis and vascular regression, permitting repair of the persistently inflamed synovium in rheumatoid arthritis. Ligands of integrin $\alpha_v\beta_3$ or antagonists acting at VEGF receptors may be capable of inhibiting angiogenesis in rheumatoid arthritis. Other agents may be required to prevent excessive vascular regression and exacerbation of synovial hypoxia.

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