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Type IV Collagen α-Chain Composition in Synovial Lining From Trauma Patients and Patients With Rheumatoid Arthritis

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Objective. **Normal synovial lining is composed of macrophage-like type A and fibroblast-like type B lining cells. This sheet-like structure lacks a basement membrane, but its intercellular substance contains some basement membrane components, including type IV collagen. We undertook this study to determine the α-chain composition of type IV collagen in normal and arthritic synovial lining, using monoclonal α-chain antibodies.**

Methods. **Samples were analyzed using avidin–biotin–peroxidase complex staining for the presence of collagen α1/2(IV), α3(IV), α4(IV), α5(IV), α6(IV), matrix metalloproteinase 2 (MMP-2), and MMP-9, and the enzyme activity was detected using gelatin zymography. Double immunofluorescence was performed for type IV collagen/MMP-9 and type IV collagen/CD68. Synovial fibroblasts were studied using quantitative reverse transcriptase–polymerase chain reaction.**

Results. **In mildly inflamed synovium from 5 trauma patients, α1/2(IV) chains were strongly stained, but α5(IV) and α6(IV) chains were weakly stained. Coding messenger RNA was shown in cultured synovial fibroblasts. Basement membranes of blood vessels contained all α(IV) chains and served as useful positive sample controls. In the synovial lining from 5 patients with rheumatoid arthritis (RA), all α-chains were absent/very weakly stained. This was coupled with numerous type A lining cells containing MMP-9 (type IV collagenase), also found in synovial fluid.**

Conclusion. **Synovial lining has a unique and very limited α-chain composition, different from that of the vascular basement membrane, which contains all α-chains. This special composition and lack of nidogen are probably of relevance for the bidirectional translining diffusion. Such tentative α-chain–dependent adhesive and transport-regulating properties seem to be deranged in RA, probably in part due to type IV collagenases produced in the lining and/or released by transmigrating or synovial fluid neutrophils.**

Synovial joints are diarthrodial moving structures in which 2 hyaline articular joint surfaces form counterfaces to each other in a low-friction and lubricated joint. Due to the avascular and noninnervated structure of the articular cartilage, the synovial membrane (and particularly the synovial lining) plays a unique role as a semipermeable barrier and as a facilitator of bidirectional translining transport. To facilitate both the nutritive and metabolic functions of the synovial membrane, subsynovial capillaries contain large fenestrations. In the arteriolar end of these capillaries, the hydrostatic pressure leads to exudation of blood plasma with all of its constituents, including dissolved oxygen. In the venular end of the synovial capillaries, the osmotic pressure exceeds the hydrostatic pressure, and metabolic waste products are taken up and transported for remote handling.

In the exchange of structural, regulatory, and metabolic molecules, translining traffic plays a key role. In many other exchange barriers, as in capillary basement membrane, this has been arranged by self assembly of laminin and type IV collagen networks, which are

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attached to each other with nidogen/entactin to form relatively solid structurally supporting mats and selectively sieving molecular filters. The synovial joint consists of the synovial membrane surrounding a synovial fluid–filled joint space. Since it needs to provide nutrition to hyaline articular cartilage, synovial intima does not contain a basement membrane similar to that which surrounds blood vessels. Instead, synovial intima or lining cell layer forms a sheet-like and compact-looking structure composed of fibroblast-like type B and macrophage-like type A lining cells (1–4). Although this is a sheet-like structure, it is not an epithelium or endothelium and does not contain a basal and coherent basement membrane. Instead, it contains some basement membrane components already mentioned above, but as an intercellular adhesive matrix (5).

We earlier characterized laminins and their integrin receptors in synovial lining in detail (6), and we found laminin-511 and laminin-521 (for the new laminin nomenclature, see ref. 7), which are recognized and bound by $\alpha6\beta1$ and $\alpha1\beta1$ integrin receptors, respectively. It was also confirmed that the synovial lining lacked laminin-332 and $\alpha6\beta4$ integrin, which are typically present in epithelial hemidesmosomes, confirming its unique structure–function architecture. In contrast, the type IV collagen α-chain composition of the normal, uninflamed lining has not been described, nor has it been determined whether this composition is altered in arthritis, in which the synovial function is deranged as part of the inflammatory loss of function. It has been found that type IV collagen does not display a homogeneous α-chain composition but is composed of 6 different α-chains. The composition of the intercellular type IV collagen in synovial lining cell–matrix composite was therefore analyzed in normal and inflamed lining, using chain-specific antibodies in immunohistochemical staining in conjunction with an analysis of type IV collagen remodeling/degrading proteinases.

MATERIALS AND METHODS

Synovial fluid and tissue samples. The study protocol was accepted by the Ethical Committee of the Helsinki and Uusimaa Hospital District. Synovial tissue samples from 10 trauma patients, 5 patients with osteoarthritis (OA), 5 patients with prosthesis loosening (synovial membrane–like interface tissue around an aseptically loosening prosthesis with an ongoing foreign body synovitis), and 10 patients with rheumatoid arthritis (RA) were used for staining unless otherwise indicated. We obtained samples of synovial fluid from 4 trauma patients with only mild inflammation (leukocyte count $<5 \times 10^9$/liter) and from 4 RA patients with relatively severe inflammation (leukocyte count 5–50 $\times$ 10^9/liter). All synovial tissue samples were snap-frozen in dry ice–precooled isopentane, embedded in OCT embedding medium (Sakura, Zoeterwoude, The Netherlands), and stored at −70°C. Samples were cut to 5-μm tissue sections, air-dried at 22°C for 1 hour, and stored at −20°C until used for staining.

Immunohistochemistry. The cryostat sections were fixed in acetone for 5 minutes at 4°C. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 30 minutes. To reveal type IV collagen immunoreactivity, the sections were denatured in acidic urea–glycine (0.1*M* glycine, 6*M* urea, pH 3.5) for 60 minutes at 4°C.

After washing with 10 m*M* phosphate buffered 140 m*M* saline, pH 7.4 (PBS), the sections were incubated in the following reagents at 22°C unless otherwise indicated: 1) normal horse serum (for monoclonal mouse anti-human antibodies), normal goat serum (for polyclonal rabbit anti-human antibodies), or normal rabbit serum (for polyclonal goat anti-human antibody) for 3 hours to block nonspecific binding sites; 2) monoclonal mouse anti-human type IV collagen α1- and α2-chain IgG1κ (1:1,000 in 0.1% bovine serum albumin [BSA] in PBS) (M3F7; Developmental Studies Hybridoma Bank, Iowa City, IA), monoclonal mouse anti-human matrix metalloproteinase 9 (MMP-9) $IgG1_\kappa$ (1:200 in 0.1% BSA in PBS) (Chemicon, Temecula, CA), polyclonal goat anti-human MMP-2 IgG (1:200 in 0.1% BSA in PBS) (R&D Systems, Minneapolis, MN), or polyclonal rabbit anti-human type IV collagen α3(IV), α4(IV), α5(IV), or α6(IV) chain IgG (1:500 in 0.1% BSA in PBS) overnight at 4°C (the α3[IV], α4[IV], and α5[IV] antibodies were a gift from Professor Jeffrey Miner [Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO] [see ref. 8], and the α6[IV] antibody was a gift from Professor Raghu Kalluri [Division of Matrix Biology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA] [see ref. 9]); 3) proper biotinylated secondary IgG (1:200 in 0.1% BSA in PBS) (Vectastain ABC Kits; Vector, Burlingame, CA); 4) avidin–biotin–peroxidase complex (ABC) (1:200 in H_2O) (Vector); and 5) a combination of 0.023% 3,3′-diaminobenzidine and 0.006% H_2O_2 for 7 minutes. For the sixth and final step, the slides were counterstained in hematoxylin, dehydrated, and mounted in Mountex (HistoLab, Gothenburg, Sweden).

Microscopic assessment. Stained slides were analyzed under 16× magnification using a light microscope (Leitz, Wetzlar, Germany) coupled with a 12-bit cooled CCD camera (Sensicam; PCO imaging, Kelheim, Germany).

Double immunofluorescence staining. After fixation in acetone at −20°C for 10 minutes, the sections (5 from trauma patients and 5 from RA patients) were thoroughly rinsed with 0.1% Triton X-100 in PBS and washed in PBS. The sections were incubated serially at 22°C in a humidified chamber in the following reagents: 1) normal goat serum (diluted 1:50; Dako, Glostrup, Denmark) for 2 hours; 2) polyclonal rabbit anti-human type IV collagen IgG (Eurodiagnostics, Apeldoorn, The Netherlands) together with monoclonal mouse anti-human MMP-9 IgG1κ or monoclonal mouse anti-human CD68 (a macrophage marker) IgG1κ (Dako) (i.e., a mixture of 2 antibodies [anti–type IV collagen and anti–MMP-9 or anti–type IV collagen and anti-CD68]) for 90 minutes; 3) a mixture of Alexa Fluor 568–conjugated goat anti-rabbit IgG and Alexa Fluor 448–conjugated goat anti-mouse IgG (diluted 1:200; Molecular Probes, Eugene, OR) for 60 minutes; and 4)

Table 1. Primer sequences used in quantitative reverse transcriptase–polymerase chain reaction and corresponding amplicon lengths

Gene	Forward primer	Reverse primer	Length, bp
COL4A1	TGGTGACAAAGGACAAGCAG	TAAGCCGTCAACACCTTTGG	268
COL4A2	TGGGATGGATGGTTTCCAAG	CCCTTGACTCCTTTGATGTG	336
COL4A3	CTGGAAGTCCTGGATCATCA	CATCAACTCCTGGGATTCCT	261
COL4A4	CCTTTGGAGATGATGGGCTA	CATCCCGGGAGTTCCTTTAT	317
COL4A5	TCGTCGCTTTAGTACCATGC	ACATTCGATGAAGGGAGCTG	367
COL4A6	TCCTGGACAAACACCAACTG	TGGAGCTCCTTCAAATCCTG	250
β-actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	295

TO-PRO-3 (diluted 1:1,000; Molecular Probes) for nuclear staining for 15 minutes. Between the steps the sections were washed in PBS, and after the last step they were also washed once in distilled water before mounting in Vectashield (Vector). All antibodies were diluted in 0.1% BSA in PBS. The stained sections were analyzed using confocal laser scanning microscopy.

Confocal laser scanning microscopy and deconvolution. Confocal microscopy was carried out using a Leica TCS SP2 system (Leica Microsystems, Mannheim, Germany) with an HCX PL APO CS 63/1.40 objective, and 568-nm and 633-nm laser excitation lines for Alexa Fluor 568 conjugate and the DNA-specific TO-PRO-3 probe, respectively. Image stacks were acquired using sequential scanning, standardized 160-nm z-sampling density, and volume depth of 3.0 μm to visualize the basal membrane constituent type IV collagen and CD68 and MMP-9 immunoreactivities.

Cell cultures. To establish synovial fibroblast lines, synovial membrane samples were obtained from 3 women with OA who had undergone total hip replacement (1 patient, age 79 years) or knee arthroscopy (2 patients, ages 60 years and 92 years, respectively). Synovial fibroblasts were established using the explant culture method. Briefly, tissue samples were minced into pieces and left overnight in RPMI 1640 medium (BioWhittaker, Liege, Belgium) containing 10% fetal bovine serum (FBS; BioWhittaker) and 10% penicillin/streptomycin. No extra ascorbic acid was added to the RPMI 1640 medium used in the experiments, so that the only ascorbic acid source used in the final medium was FBS. However, it is to be noted that type IV collagen α-chain synthesis was studied upstream of ascorbic acid–dependent hydroxylation and crosslinking of the peptidyl proline and the peptidyl lysine (i.e., at the messenger RNA [mRNA] level).

The next day, the medium was changed and the concentration of antibiotics was decreased to 1%. The medium was changed twice a week, and after ~60% of the dish area was covered with a monolayer of cells, the tissue pieces were removed and the cultures were allowed to grow to confluence. When such cells from the third through fifth passages are stained using prolyl 4-hydroxylase as a fibroblast marker (10) and CD163 (11) as a macrophage marker, the proportion of the prolyl 4-hydroxylase–positive spindle-shaped (i.e., fibroblast-like) cells is >99%, whereas the proportion of the CD163-positive macrophages is <1% (data not shown). In addition, these cells show other characteristics of fibroblasts, since they are Hsp47, vimentin, and fibronectin positive (data not shown). For these stainings, the following antibodies were used in indirect immunofluorescence staining as described above: anti-CD163 (diluted 1:100) (10D6; Novocastra, Newcastle-upon-Tyne, UK) and antifibroblast antibody (diluted 1:200) (M0877; Dako). The Z1 Coulter Particle Counter (Beckman Coulter, Fullerton, CA) was used for cell counting.

Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). For stimulations, 10^5 cells/well were grown to confluence in 6-well plates. Cells from 2 parallel wells were used for RNA extraction. The cells were stimulated for 48 hours with 0.1, 1, and 10 ng/ml recombinant human tumor necrosis factor α (TNFα) or recombinant human interleukin-1β (IL-1β) (both from R&D Systems). Total RNA from cells was isolated using TRIzol reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. RNA quality was confirmed with ethidium bromide–stained 1% agarose gel. The mRNA was isolated with magnetic Oligo(dT)25 polystyrene beads (Dynal, Oslo, Norway). Messenger RNA concentration was measured spectrophotometrically, and complementary DNA (cDNA) was synthesized from 50 ng of sample mRNA using oligo$(dT)_{12\text{-}18}$ primers and SuperScript enzyme, followed by RNase H treatment (SuperScript Preamplification System; Invitrogen). Quantitative RT-PCR was run using 4.8 ng first-strand cDNA and 0.5 m*M* primers in LightCycler PCR mix in a LightCycler PCR machine (Roche, Mannheim, Germany). Quantitative RT-PCR runs were repeated twice with each sample.

For primers, the sequences were searched with the National Center for Biotechnology Information (NCBI) Entrez search system, and sequence similarity search was done using the NCBI Blastn program. The primers were designed, if possible, to produce an amplicon that extended over 2 different exons (Table 1). For the quantitative RT-PCR standard curve, the gene of interest was amplified in the PCR, extracted from an agarose gel, and cloned into the pCRII-TOPO vector (Invitrogen). After identification of the plasmid by restriction enzyme analysis and sequencing, the concentration was determined spectrophotometrically, and serial dilutions were prepared for quantitative RT-PCR analysis. The copy numbers of mRNA were determined with 2 separate runs for all samples and normalized against 1×10^6 copies of the housekeeping β-actin gene.

Gelatin zymography. Synovial fluid samples (n = 8) were analyzed for gelatinase activity using a gelatin-containing sodium dodecyl sulfate polyacrylamide gel under nonreducing conditions. Gelatinase zymography standards were used (Chemicon). Fifteen-microliter aliquots of synovial fluid samples diluted 1:50 with PBS were run on a 7.5% gelatin-containing polyacrylamide gel (Bio-Rad, Hercules, CA) at 200V for 1 hour, after which the gel was carefully removed

from the plates and washed in 0.05*M* Tris HCl, 0.02% (weight/volume) NaN_3, pH 7.5, and 2.5% Tween 80 for 30 minutes with 3 washings in between. The gel was washed in 0.05*M* Tris HCl, 0.02% NaN_3, pH 7.5, 2.5% Tween 80, 1 μM $ZnCl_2$, and 5 m*M* $CaCl_2$ for 30 minutes at 22°C. Finally, the gel was incubated in 0.05*M* Tris HCl, pH 7.5, 0.02% NaN_3, 1 μM $ZnCl_2$, and 5 m*M* $CaCl_2$ overnight at 37°C and then stained with 1.2 m*M* Coomassie brilliant blue (Serva, Heidelberg, Germany) for 1 hour. Destaining was done with destaining solution containing 70% water, 20% methanol, and 10% glacial acetic acid. The bands were visible as light bands against a dark blue background.

RESULTS

Immunohistochemistry findings. In immunohistochemical staining, α1/2(IV) chains showed strong labeling in synovial membrane samples from trauma patients, and α5(IV) and α6(IV) chains were also seen in synovial lining from trauma patients, although their labeling was weak compared with synovial α1/2(IV) chains and vascular basement membrane α5(IV) and α6(IV) chains (Figure 1). Immunohistochemical staining did not disclose any α3(IV) or α4(IV) chain labeling. Results similar to those seen in synovial membrane in trauma patients were also observed in synovial membrane in OA and in synovial membrane–like interface tissue around aseptically loosened total hip replacement implants (Figure 2). In contrast to the control samples, in synovial lining in RA patients, not only α5(IV) and α6(IV) chains but also α1/2(IV) chains were only weakly expressed (Figure 1). Staining with the irrelevant immunoglobulins of the same class and subtype, using the same concentrations as were used for immunolabeling, confirmed the specificity of the staining results (data not shown).

Double staining and confocal laser scanning microscopy. Synovial lining in the control synovial membrane samples obtained from trauma patients labeled strongly for type IV collagen. Relatively few of the control synovial lining cells were CD68-positive macrophage-like type A lining cells (Figure 3C) or MMP-9 positive (Figure 3A). In contrast, the type IV collagen staining pattern in the rheumatoid synovial lining was weak and mottled (Figures 3B and D), and the rheumatoid synovial lining contained relatively numerous macrophage-like CD68-positive type A lining cells, which had arranged themselves into pseudostratified multiple layers (Figure 3D). Many of these rheumatoid synovial lining cells were also positive for MMP-9 (Figure 3B).

Quantitative RT-PCR findings. Each of the 3 synovial fibroblast cell lines was grown, stimulated, and analyzed separately. In spite of significant differences in

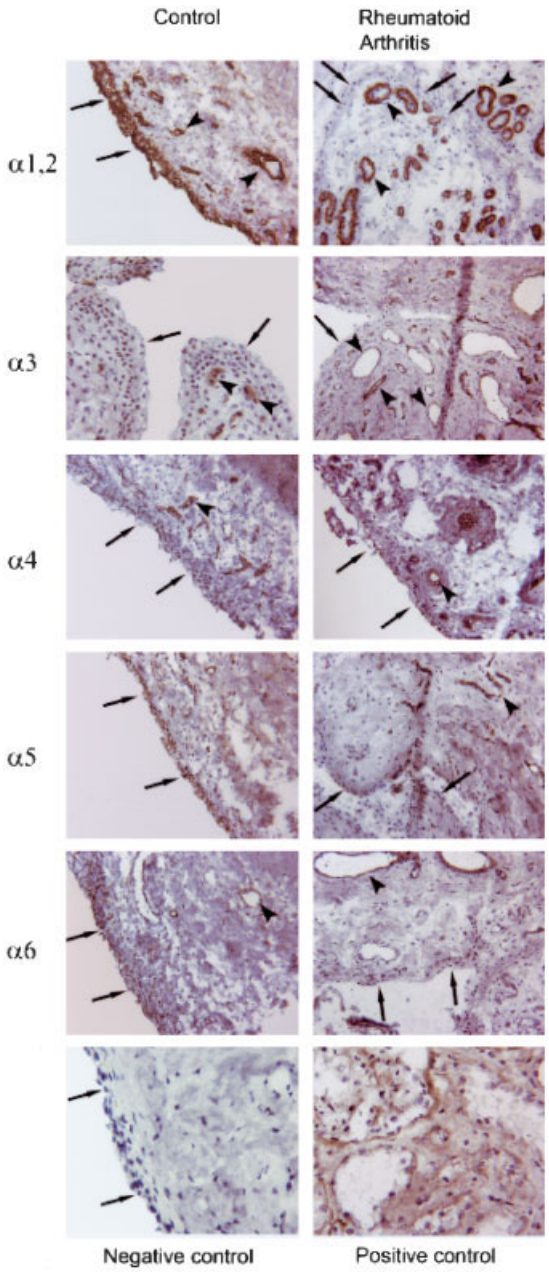


Figure 1. Avidin–biotin–peroxidase complex staining of type IV collagen α-chains in control synovial membrane samples obtained from trauma patients and in inflammatory synovial membrane samples obtained from rheumatoid arthritis patients. Strong α1/2(IV) chain labeling and weaker α5(IV) and α6(IV) chain labeling is seen in the control synovial lining (**arrows**), whereas α3(IV) or α4(IV) chains were not found. In contrast, rheumatoid synovial lining is only weakly labeled (**arrows**). Strong labeling of all of the α(IV) chains in the vascular basement membranes (**arrowheads**) serves as an internal positive staining control demonstrating that the staining was technically successful. The positive (sample) control is a section of kidney tissue that contains all the collagen α-chains. The negative (staining) control is a synovial tissue section stained using nonimmune rabbit IgG instead of any primary type IV collagen α-chain–specific antibodies (original magnification $\times$ 10).

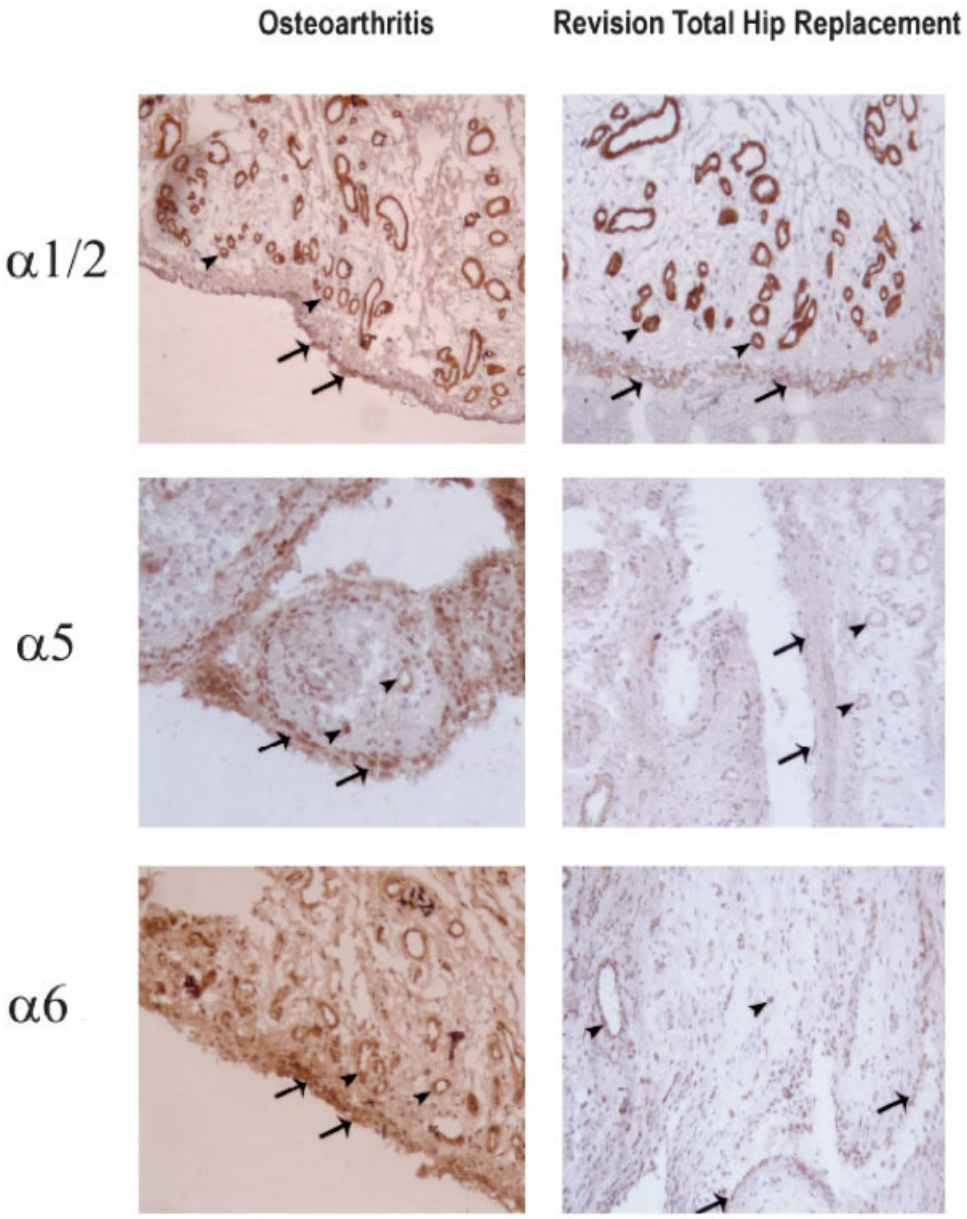


Figure 2. Avidin–biotin–peroxidase complex staining of type IV collagen α-chains in control synovial membrane samples obtained from osteoarthritis patients and in inflammatory synovial membrane–like interface tissue obtained from patients with aseptically loosened total hip replacement implants. Results are rather similar to those seen in synovial membrane in trauma patients, so that the lining displays strong α1/2(IV) chain labeling and weaker α5(IV) and α6(IV) chain labeling (**arrows**). However, in the synovial membrane–like interface tissue samples (representing foreign body synovitis) from patients who had undergone revision total hip replacement, the lining was slightly weakened. Vascular basement membrane α(IV) chains (**arrowheads**) serve as internal positive controls. Staining of the lining can be compared with the staining of the vascular basement membranes (original magnification $\times$ 10). Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

the age of patients and, most likely, the genetic variations among them, the actual variations between individual cell lines were minor, and the results are presented as the mean $\pm$ SD. Quantitative RT-PCR disclosed that cultured synovial fibroblasts contained mRNA coding for α1(IV) and α2(IV) chains; α4(IV) and α6(IV) chain mRNA were also present, although at lower copy numbers, while copy numbers for α5(IV)

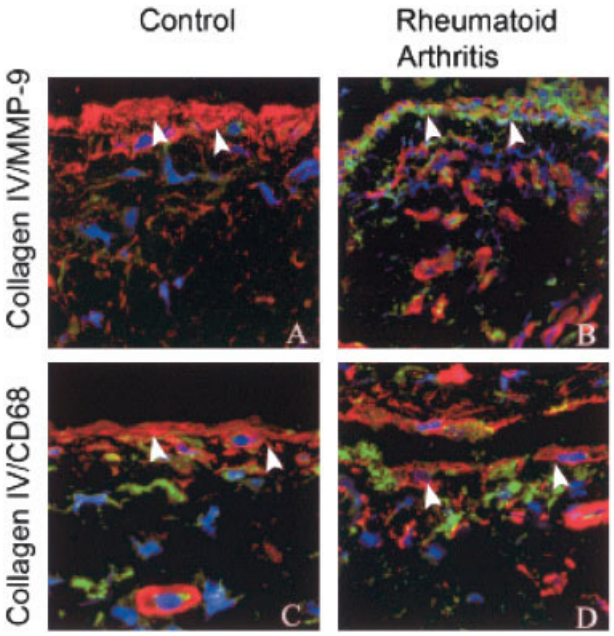


Figure 3. Indirect immunofluorescence double labeling. Type IV collagen (collagen IV) is labeled red in all panels. Colabeling of type IV collagen with matrix metalloproteinase 9 (MMP-9) (type IV collagenase) and colabeling of type IV collagen with macrophage marker CD68 are both shown in green. TO-PRO-3–labeled nuclei are shown in blue. Control synovial lining from a trauma patient contains type IV collagen–rich intercellular matrix in the lining (**A** and **C**), but very few of the lining cells are MMP-9 positive (**A**) and/or CD68 positive (**C**). In contrast, rheumatoid synovial lining contains very little intercellular type IV collagen (**B** and **D**), but many of the lining cells are MMP-9 positive (**B**) and/or CD68 positive (**D**). (Original magnification × 20).

chain mRNA and particularly α3(IV) chain mRNA were very low (Figure 4). Recombinant human TNFα and particularly recombinant human IL-1β decreased α(IV) chain mRNA copy numbers, but at the 0.1–10 ng/ml concentrations used, this effect did not show any clear dose dependency (Table 2).

Gelatin zymography findings. Gelatin zymography of synovial fluid samples obtained from trauma patients with only mild inflammation (leukocyte count $<5 \times 10^9$/liter) showed MMP-2 but no MMP-9 (Figure 5). In contrast, synovial fluid from RA patients with relatively severe inflammation (leukocyte count 5–50 × 10^9/liter) contained much more MMP-2 and MMP-9 (Figure 5). The same synovial fluid samples also contained some constitutively expressed 72-kd MMP-2 (Figure 5).

DISCUSSION

It has been known for some time that the interstitial matrix between fibroblast-like type B and macrophage-like type A synovial lining cells contains most, if not all, components of the basement membrane (12). The laminin chain composition of this intercellular lining matrix has been described earlier (6), but the α-chain composition of type IV collagen in synovial lining has not. Since this special extracellular lining matrix is of particular interest due to its role as a leukocyte barrier and in cartilage nutrition, the α-chain composition of the synovial lining was analyzed in the present study using chain-specific antibodies. This disclosed the presence of α1/2(IV), α5(IV), and α6(IV) chains in control synovial lining. In contrast, α3(IV) and α4(IV) chains were not found in immunohistochemical staining. These conclusions were nicely confirmed by an internal positive sample control (i.e., the positive staining of the vascular basement membrane in the very same samples). For example, the vascular basement membrane in normal synovial lining stained strongly for α4(IV) chain, but none of that was seen in the synovial lining.

These findings were confirmed by using cultured synovial fibroblasts analyzed using quantitative RT-PCR, although this sensitive method also disclosed traces of mRNA coding α3(IV) and even α4(IV) chains. Apparently, the mRNA level and/or the translation to the corresponding α3(IV) or α4(IV) protein chains was so slight (or perhaps even absent) that it could not be seen in staining experiments, except in the vascular basement membranes, from where the chains could derive. It is also noteworthy that the antibody used for staining of the major α-chains α1(IV) and α2(IV) recognized both of them (i.e., did not differentiate between them). However, α-chain–specific quantitative RT-PCR

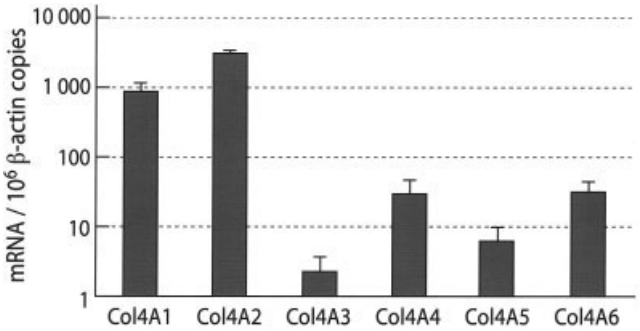


Figure 4. Quantitative reverse transcriptase–polymerase chain reaction analysis of type IV collagen α-chain mRNA copy numbers per 10^6 β-actin copies in cultured synovial fibroblasts. To show the presence of the α(IV) chains with low copy numbers, the results are presented on a logarithmic scale. Values are the mean and SD. Col4A1 = mRNA copy numbers of the gene coding for type IV collagen α1 chain.

Table 2. Effect of 0.1–10 ng/ml recombinant human IL-1β and recombinant human TNFα on α(IV) chain mRNA copy numbers in synovial fibroblasts compared with unstimulated synovial fibroblast controls*

	α1(IV)	α2(IV)	α3(IV)	α4(IV)	α5(IV)	α6(IV)
Control	100	100	100	100	100	100
IL-1β, ng/ml						
0.1	81	70	49	40	71	55
1	68	64	62	45	68	45
10	77	65	59	36	54	29
TNFα, ng/ml						
0.1	74	74	85	91	88	102
1	79	73	100	109	83	51
10	67	67	109	83	72	111

* Values are the mean from 3 separate experiments. IL-1β = interleukin-1β; TNFα = tumor necrosis factor α.

disclosed that both α1(IV) and α2(IV) mRNA are found in cultured synovial fibroblasts, indicating that both are present in synovial lining and confirming that they are the major type IV collagen α-chains in normal synovial lining.

In contrast to normal synovial lining, rheumatoid synovial lining contained very few (or even lacked) α1/2(IV), α5(IV), and α6(IV) chains in staining or, alternatively, their expression was below the detection threshold of the method used. It can be concluded that levels of type IV collagen α-chain proteins are very low in rheumatoid synovial lining, since the ABC staining method used is very sensitive (13). This indicates that binding of type A and B cells to the lining cell layer is probably relatively loose in the hyperplastic rheumatoid synovial lining compared with the healthy synovial lining. Indeed, detached synovial lining cells can be seen in the cytologic analysis of rheumatoid synovial fluid (14). This loosening of the intercellular matrix in RA might in part contribute to synovial effusions, which are characteristically found in this and other inflammatory joint diseases. For example, the mean ± SD knee joint volume has been estimated to be 6.7 ± 2.3 ml in normal subjects, compared with 13.6 ± 7.4 ml in patients with mild OA and 24.2 ± 16.3 ml in patients with active OA (15), but it is often much higher in RA patients (16).

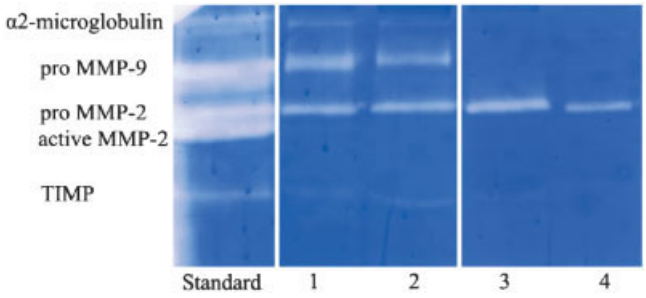


Figure 5. Gelatin zymography of matrix metalloproteinase 9 (MMP-9) and MMP-2 in synovial fluid samples. Lanes 1 and 2, Synovial fluid samples from rheumatoid arthritis patients with severe inflammation; lanes 3 and 4, synovial fluid (control) samples from trauma patients with only mild inflammation. Only rheumatoid synovial fluid is strongly positive for 92-kd MMP-9, while the control samples contain only proMMP-2. TIMP = tissue inhibitor of metalloproteinases. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

It was thought that this report would be strengthened by parallel studies of non-RA inflammatory disease, particularly to see whether these changes are specific for RA. For this purpose, we studied synovial membrane from OA patients as well as synovial membrane–like interface tissue (representing foreign body synovitis) around loosening total hip replacement implants. The findings in these 2 conditions were rather similar to those seen in synovial membrane in trauma patients. However, staining of the lining was slightly diminished in synovial membrane–like interface tissue samples obtained from patients who had undergone revision total hip replacement. Therefore, it is still likely that the disappearance of the interstitial type IV collagen in RA results from chronic and active inflammation (e.g., the heavy neutrophil traffic across the lining cell layer) rather than from some other disease-specific features per se. Unfortunately, we were not able to collect psoriatic arthritis synovial tissue for comparison to test this hypothesis.

The reason for the diminished type IV collagen α-chains in the synovial lining in RA is not known. However, some suggestions can be made based on the present findings. Staining of the synovial lining using the monocyte/macrophage marker CD68 disclosed that the proportion of synovial lining macrophage-like type A

cells is greatly increased in RA. This has also been described before (3) and may reflect the role of the villous and hyperplastic lining in the handling of apoptotic synovial fluid neutrophils in RA. For example, if a knee joint contains 50 ml of synovial fluid containing 50×10^9 neutrophils/liter, 1.25×10^9 apoptotic neutrophils have to be handled during 1 day if the neutrophils are expected to have a 2-day lifespan after they have left the intravascular compartment and entered the synovial fluid. Such macrophage-like type A lining cells do not produce or contain type IV collagen, which in the synovial lining is produced by fibroblast-like type B lining cells. Therefore, the hyperplastic synovial lining, which contains an increased proportion of macrophage-like type A lining cells, contains relatively few type IV collagen–producer cells.

We hypothesized that a second reason for the diminished type IV collagen α-chain expression in the rheumatoid synovial lining might relate to inflammatory stimulation of the rheumatoid synovial lining fibroblast-like type B cells. To assess this more closely, rheumatoid synovial fibroblasts were stimulated with proinflammatory doses of IL-1β or TNFα. Both stimulations decreased type IV collagen α-chain mRNA levels in the stimulated synovial fibroblasts. The range of the concentrations of the cytokines was selected so that they corresponded to physiologically relevant values. However, only stimulation with IL-1β tended to have a dose-response effect, while stimulation with TNFα did not, indicating that type IV collagen α-chain synthesis is particularly sensitive to the effect of TNFα, since 0.1 ng/ml was already effective. In any case, both of these proinflammatory cytokines decreased type IV collagen α-chain synthesis in rheumatoid synovial fibroblasts. In vivo, they are likely to act in concert and together with other locally produced inflammatory cytokines. Therefore, such combined inhibition by proinflammatory cytokines may also contribute to the diminished local synthesis of type IV collagen in synovial lining. Interestingly, it seems that all type IV collagen α-chains were diminished by these proinflammatory cytokines.

The third possible reason for the diminished type IV collagen expression in the rheumatoid synovial lining may be increased local and/or extrinsic type IV collagenase activity. There was a great increase in the level of 92-kd MMP-9 (gelatinase B) in rheumatoid synovial lining, particularly in the CD68-positive, macrophage-like type A lining cells. MMP-9 expression indicates simultaneous activation of the rheumatoid synovial lining, since MMP-9 expression is inducible by proinflammatory factors, and MMP-9 positivity indicates an activated state of the cell. In addition, according to gelatin zymography, inflammatory rheumatoid synovial fluid contained increased levels of MMP-9. Although none of it was activated as seen in synovial fluid per se, it might be that the activated MMP-9 was tightly bound to its intercellular matrix in the lining and was therefore not found in its free activated form in the fluid phase.

Gelatin zymography showed the relative amounts of MMP-2 and MMP-9 in synovial fluid from RA patients with severe inflammation compared with synovial fluid from trauma patients with mild inflammation. Since no synovial fluid samples from normal subjects were tested, it is not possible to compare these results directly with such samples. However, neutrophils form the major source of synovial fluid MMP-9. Because the healthy synovial fluid contains $<0.2 \times 10^9$ leukocytes per liter, and $<25\%$ of them are neutrophils (17), it is likely that the amount of MMP-9 would be even lower in healthy synovial fluid than in our synovial fluid samples from trauma patients with mild inflammation (although it was already below the detection limit of the zymography in the samples from trauma patients). Therefore, both locally produced MMP-9 and MMP-9 released by neutrophils traversing the lining or residing in the synovial fluid may contribute to local degradation of type IV collagen and thus to the diminished expression of type IV collagen α-chains in the rheumatoid synovial lining.

In conclusion, the present study confirms earlier pioneering findings demonstrating the presence of type IV collagen in synovial lining (18,19). It also extends these earlier studies by demonstrating that particularly α1(IV) and α2(IV) chains are present. This shows that the type IV collagen α-chain profile is very limited in synovial lining compared with synovial blood vessels, which contained all type IV collagen α-chains. Type IV collagen α-chain expression is greatly diminished in rheumatoid synovial lining, probably due to multiple factors, all of which contribute to the same outcome. These factors include an increased proportion of type IV collagen nonproducers (macrophage-like type A lining cells), increased local degradation by MMP-2 and MMP-9 locally synthesized and/or released (by synovial lining and fluid cells), and diminished local type IV collagen production as a result of inflammatory loss of function (caused by TNFα and IL-1β).

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AUTHOR CONTRIBUTIONS

Dr. Konttinen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Virtanen, Konttinen.

Acquisition of data. Poduval, Sillat, Beklen, Kouri.

Analysis and interpretation of data. Poduval, Sillat, Beklen, Kouri, Konttinen.

Manuscript preparation. Poduval, Sillat, Beklen, Kouri, Virtanen, Konttinen.

Statistical analysis. Poduval, Sillat, Konttinen.

Organization. Konttinen.

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