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ORIGINAL ARTICLE

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Loss of collagen type IV in rheumatoid synovia and cytokine effect on the collagen type-IV gene expression in fibroblast-like synoviocytes from rheumatoid arthritis

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Abstract Collagen type IV is a structural matrix protein which contributes to the structural organization of the synovia. In order to characterize the distribution of this protein in synovia with chronic synovitis, collagen type IV was detected by immunochemistry in normal synovia and in synovia from patients with osteoarthritis (OA) and rheumatoid arthritis (RA). A decrease of collagen type IV was observed in synovial layers of rheumatoid synovia, which statistically correlated with the grade of inflammation and with the thickness of the synovial layer. In vitro, we found no differences in the gene expression of collagen type IV in cultures of fibroblast-like synoviocytes (FLS) derived from OA and RA using a reverse-transcriptase polymerase chain reaction. Nevertheless, we observed a downregulating effect of tumor necrosis factor-α and interleukin (IL)-1β on the gene expression of collagen type IV only in FLS isolated from patients with RA. The effect of IL-1β was dose dependent. In summary, we observed an inflammation-associated decrease of collagen type IV in the synovial layer of rheumatoid synovia. Inflammatory cytokines may play a role in regulating the synthesis of collagen type IV in the rheumatoid process in vivo.

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Introduction

Extracellular matrix proteins play an important role in maintaining the integrity of tissues [2, 17]. Although normal synovia lacks a basement membrane, there is a clearly defined distribution of extracellular matrix proteins such as collagen type IV and laminin in the synovia. This suggests that this network of extracellular matrix components contributes to the organization of the synovia [19, 23, 24]. One of the most striking aspects of chronic synovitis is the remodeling of the connective tissue matrix. The concomitant degradation of the normal synovial tissue and the replacement of this tissue by inflammatory cells and fibrous tissue results in joint destruction and loss of function. Potential effectors of this destructive process are synovial lining cells (SLCs), which have been shown to synthesize extracellular matrix proteins [28] and proteolytic enzymes [18]. In chronic synovitis, cytokines may induce a degradative phenotype in SLCs [15]. In situ and in vitro studies have shown that fibroblast-like synovial cells (FLSs) synthesize collagen type IV [19, 28]. Since collagen type IV is regarded as a major structural component of the basement membrane, we investigated the in situ distribution of this structural protein using immunochemistry in synovia with chronic synovitis from patients with osteoarthritis (OA) and rheumatoid arthritis (RA). In order to characterize the regulating factors of the remodeling process of the extracellular matrix in vivo, we detected the effect of cytokines on the collagen type-IV gene expression using reverse-transcription polymerase chain reaction (RT-PCR) in FLS in vitro from patients with OA and RA.

Materials and methods

Synovial tissue

Synovial tissues were obtained at arthroplasty from different joints of 23 patients with RA and 16 patients with OA (according to the criteria of the "American College of Rheumatology", formerly the "American Association of Rheumatology" [4, 6]). Control specimens were obtained at autopsy from two patients without arthritis who died of unrelated causes. Autopsy specimens were taken within 6 h of death. Control specimens, 19 specimens of patients with RA and 12 synovia specimens of patients with OA, were quick-frozen in liquid nitrogen and stored at –70°C until the immunostaining procedure was performed. Laboratory tests, erythrocyte sedimentation rate (ESR) and rheumatoid factor are reported in Table 1. The specimens from four patients with OA and from four patients with RA were processed immediately after excision and prepared for FLS culture.

Reagents

The anti-collagen type-IV monoclonal antibody (mAb) CIV22 (IgG1) was purchased from Dako (Hamburg, Germany). A polyclonal biotinylated sheep antibody to mouse immunoglobulin (Ig; reactive with all mouse and rat isotypes) and a streptavidin-biotinylated peroxidase complex were purchased from Amersham (High Wycombe, UK). 3-Amino-9-ethylcarbazole (AEC) and *N′N*-dimethylformamide (DMF) were obtained from Sigma Chemical (St. Louis, Mo.). An anti-cytomegalovirus antibody (clone CCH2; IgG1) was used as a negative control and was obtained from Dako (Glostrup, Denmark).

Immunostaining procedure

Serial frozen sections of approximately 1 cm^2 in area and 5 µm in thickness were air-dried overnight, fixed in acetone for 10 min at room temperature, and immunostained immediately or after stor-

Table 1 Inflammation score, clinical, and immunostaining data in synovial lining cells of normal, osteoarthritis (OA), and rheumatoid arthritis (RA) synovia. Scoring of cell reaction: 4+, 75–100% cells positive; 3+, 50–75% cells positive; 2+, 25–50% cells positive; 1+, 1–25% cells positive; (), low level of antigen expression; –, all cells negative. Scoring of synovial lining layers: 1, 1–3 cell layers; 2, 4–5 cell layers; 3, 6–8 cell layers; 4, 9 cell layers. *ESR* erythrocyte sedimentation rate, *RF* rheumatoid factor, *NSAID* non-steroidal anti-inflammatory drug, n.d. not determined

	ESR	RF	Antirheumatic treatment	Site of synovia	Inflammation score	Synoviocyte hyperplasia	Collagen type IV
Normal synovia							
Sample 1	–		–	Knee	3	1	4+
Sample 2	–		–	Knee	3	1	4+
OA synovia							
Sample 3	7		No	Knee	5	2	3+
Sample 4	2		No	Knee	8	2	(1+)
Sample 5	20		No	Hip	7	1	4+
Sample 6	2		No	Hip	11	1	3+
Sample 7	30		No	Hip	4	1	3+
Sample 8	3		No	Hip	4	1	3+
Sample 9	20		No	Hip	6	1	3+
Sample 10	n.d.		No	Knee	4	1	3+
Sample 11	6		NSAID	Hip	8	1	(3+)
Sample 12	2		NSAID	Hip	7	1	(4+)
Sample 13	18		No	Hip	6	2	(2+)
Sample 14	3		NSAID	Hip	4	1	3+
mean value ±SD					6.2 (2.1)	1,2 (0.4)	2.9 (0.7)
RA synovia							
Sample 15	18	+	Gold	Knee	7	1	(2+)
Sample 16	22	+	Gold	Wrist	11	1	1+
Sample 17	9	n.d.	Methotrexate	Wrist	15	4	–
Sample 18	20	+	Methotrexate	Wrist	9	2	4+
Sample 19	35	–	Methotrexate	Wrist	9	1	(2+)
Sample 20	n.d.	+	Gold	Knee	11	1	1+
Sample 21	16	+	NSAID	Wrist	12	1	2+
Sample 22	44	+	Methotrexate	Knee	9	2	–
Sample 23	40	n.d.	Methotrexate	Hip	8	2	(2+)
Sample 24	34	+	Gold	Knee	13	2	(1+)
Sample 25	4	–	Methotrexate	Hip	4	3	(2+)
Sample 26	54	+	Methotrexate	Knee	6	1	(2+)
Sample 27	21	–	Methotrexate	Elbow	15	1	(1+)
Sample 28	17	n.d.	Methotrexate	Knee	10	2	3+
Sample 29	33	–	NSAID	Wrist	12	4	–
Sample 30	80	+	Penicillamine	Knee	15	4	–
Sample 31	20	n.d.	Gold	Knee	15	4	–
Sample 32	14	+	Methotrexate	Hip	10	2	(1+)
Sample 33	20	n.d.	Penicillamine	Knee	8	1	3+
Mean value ±SD					10.6±3.2	2.1±1.1	1.3±1.13

age at –20°C for 1–3 weeks. Immunostaining procedures were carried out according to standard methods as previously described [25]. Briefly, for immunohistochemistry, following rehydration with phosphate-buffered saline solution (PBS; pH 7.5), the frozen sections were incubated for 1 h with primary mAbs. The primary mAb was used in a protein concentration of about 5 µg/ml PBS. The sections were then incubated with biotinylated anti-mouse antibody (20 µg/ml protein) and a streptavidin–biotin–peroxidase complex (10 µg/ml protein) for 30 min, respectively. All incubation steps were carried out in a humid chamber at room temperature. Between each incubation step, the sections were rinsed twice in PBS. Using AEC as the chromogen (0.4 mg/ml in 0.1 mol/l acetate buffer, pH 5.0, with 5% DMF and 0.01% hydrogen peroxide for about 20 min), the peroxidase reaction caused an intense-red precipitate. The sections were then rinsed in tap water, counterstained with Harris' hematoxylin, and mounted with glycerol gelatin. Each series of frozen sections contained a negative control without the primary reagent and the control anti-cytomegalovirus antibody. No staining was observed in the control slides except for scattered granulocytes whose endogenous peroxidase was not blocked in order to permit optimal antigenicity. This reactivity was disregarded during evaluation. In tissue sections, strongly stained endothelial cells served as intrinsic positive controls.

Controls and evaluation of antigen expression

SLC staining in tissue sections was evaluated in a semi-quantitative manner: ''+'' indicates strong intensity of the detected antigen; ''(+)'' is regarded as weak intensity of antigen detected. The absence of antigen was symbolized as ''–''. A simple semi-quantitative statement was made for the staining intensity within SLCs: ''4+'', meaning 75–100% cells positive; ''3+'', meaning 50–75% cells positive; ''2+'', meaning 25–50% cells positive; and ''1+'', meaning 1–25% cells positive.

Degree of inflammation

Hematoxylin-eosin-stained sections from each synovial specimen were scored for the degree of inflammation by two independent observers, according to Rooney et al. [27]. SLC depth was determined, and the results were recorded as 1 (1–3 cell layers), 2 (4–5 cell layers), 3 (6–8 cell layers), and 4 (=9 cell layers). Proliferating blood vessels were determined by immunostaining using anti-von Willebrand Factor (Dako) and recorded as 1+ [0–5 vessels/high power field (HPF)], 2+ (5–10 vessels/HPF), 3+ (10–20 vessels/HPF), and 4+ (>20 vessels/HPF). The size of lymphoid aggregates on stained sections was recorded as 0+ (no aggregates), 1+ (1–20 cells/HPF), and 2+ (>20 cells/HPF). The number of vessels showing perivascular infiltrates was determined for the final score and regarded as 1+ (1–25% of the vessels involved), 2+ (25–50% of the vessels involved), 3+ (50–75% of the vessels involved), and 4+ (75–100% of the vessels involved). The degree of fibrosis and the presence of necrosis were assessed. Sections containing less than 10% fibrous tissue in the membrane sublining layers were considered normal and graded 1. Sections containing 10–50% fibrous tissue were scored as 2+, whereas sections with 50% fibrous tissue were graded as 3+. The absence of necrosis was scored as 0. The presence of necrosis was scored as 2+. The scores of the individual items were added, and the sum was used as an estimate of the overall intensity of inflammation using histological criteria (range of possible values 3–19).

FLS isolation

In vitro cultures of FLS were obtained using standard methods, as previously described [1]. The tissue was minced into small pieces and digested with collagenase type Ia (Sigma Chemical) in serum-free basal Iscove medium (Seromed-Biochrom, Berlin, Germany). The samples were washed and suspended in basal Iscove medium, supplemented with 10% fetal calf serum (FCS, Bio Pro, Karlsruhe, Germany) and penicillin–streptomycin–amphotericin B (10 U/ml, 10 mg/ml, and 0.25 mg/ml). Finally, isolated cells were cultured in a humidified 5% carbon dioxide atmosphere. After overnight culture, non-adherent cells were removed. Fresh medium was added and the incubation continued. At confluence, cells were trypsinized, split at a ratio of 1:3 and re-cultured. The medium was changed twice weekly. At the end of in vitro incubation, FLS cultures were washed with PBS, detached with ethylene diamine tetraacetic acid (EDTA; 0.02%) and centrifuged at 1000 rpm for 5 min. The cultured cells comprised a homogeneous population of FLSs with respect to morphological and immunocytochemical criteria (<1% CD11b+, <1% CD11a+, <1% CD11c+, <1% CD53+, <1% CD3+, <1% factor VIII+).

FLS cultures for RT-PCR

Collagen type-IV gene expression was investigated in 3 OA-FLS and 3 RA-FLS cultures at different culture times (from passages 1 to 5) and in two other FLS-cultures (1 OA-FLS and 1 RA-FLS from passages 3 to 9) after stimulation with interleukin (IL-1β; Genzyme; Cambridge, Mass.; 40 U/ml, 100 U/ml, and 1000 U/ml), tumor necrosis factor (TNF)-α (Knoll; Ludwigshafen, Germany; 10 ng/ml), and transforming growth factor (TGF)-β (Boehringer; Mannheim, Germany; 10 ng/ml). FLS cultures were incubated for 72 h in serum-free conditions with these cytokines, used alone or in combination (IL-1β and TNF-α, IL-1β and TNF-α, TGF-β and IL-1β), or with medium alone as a negative control. Each FLS culture (4–5×10^5 cells) was lysed using a guanidinium thiocyanate buffer and stored at –70°C until RNA extraction.

Preparation of RNA and complementary (c)DNA synthesis

Total RNA was extracted from cultured FLSs using the method described by Chomczynski and Sacchi [8], based on a guanidinium thiocyanate buffer. The amount of RNA obtained was photometrically determined and 0.2 µg total RNA used for cDNA synthesis with the following reagents (total volume of 25 µl): 8 µM random hexamers (Pharmacia, Freiburg, Germany) 1 mM deoxyribonucleoside triphosphate (dNTP; Promega, Madison, Wis.), 20 U AMV reverse transcriptase (Promega), 40 U RNasin (Promega), 50 mM KCl, 5 mM Tris-HCl, and 10 mM $MgCl_2$. After incubation for 1 h at 42°C, newly synthesized cDNA was stored at –20°C until further use.

Semi-quantitative PCR analysis of collagen type-IV mRNA expression

Primers of collagen type-IV cDNA were devised from published sequences [14, 30]: sense 5′-GCTCACCAGGACCAGTGGGT-3′, antisense 5′-TCACCTTTAGGTCCTGGCTG-3′. Primers for cDNA of the housekeeping gene β2-microglobulin were used that closely matched the melting temperature of collagen type-IV primers: sense 5′-CTCGCGCTACTCTCTCTTTCT-3′, antisense 5′-TGTCGGATTGATGAAACCCAG-3′.

The PCR reaction of a 2-µl cDNA aliquot contained: 2 U *Taq* polymerase (Promega), 0.83 µM of the respective PCR primers, 3 mM $MgCl_2$, 200 µM dNTP, 1×PCR buffer (Promega; total volume of 50 µl). Thirty PCR cycles were performed at temperatures of 94°C, 60°C, and 72°C, each for 1.5 min. Previous experiments had shown that, under these conditions, the PCR reaction was in the exponential phase. PCR products were separated according to size in 2% agarose gel and transferred onto a nylon membrane (Boehringer). PCR from collagen type-IV cDNA yielded a 310-bp product.

To confirm the identity of the amplification products and to determine the amounts of PCR products, nylon filters were simulta-

neously hybridized with the following 3′-digoxigenin-labelled oligonucleotide probes specific to internal collagen type IV (5′-AAAGGTGATAAGGGGGATGTCGGTCTG-3′) and β2-microglobulin sequences (5′-ATTCTCTGCTGGATGACGTGAGTAAACCTG-3′). Prehybridization with a buffer described by Church and Gilbert [9] containing 5×SSC (sodium saline citrate), 2.0% blocking solution (Boehringer), 0.1% *N*-lauroylsarcosyl (Serva, Germany), 7% sodium dodecylsulphate (Sigma Chemical), 50 mM sodium phosphate, pH 7.0, and 50 µg/ml yeast tRNA (Boehringer) was performed for 2 h in a hybridization flask with continuous rolling (Bachofer, Germany). Subsequent hybridization was performed for approximately 15 h using 2.5 ml per 100 cm^2 filter surface of the same buffer with 10 pmol/ml digoxygenin (DIG)-labeled oligonucleotide probes specific to the β2m and the collagen type-IV gene. Washing conditions and chemoluminescence reaction with CSPD (disodium 3-(methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate) as substrate were performed using instructions and reagents of a commercially available chemoluminescence detection kit (Boehringer). Emission of light was detected by exposition of X-ray films. Signals on the exposed films were quantified after scanning (Macintosh Color One Scanning) using IMAGE 1.41 software (National Cancer Institute, Bethesda, Md.). Relative collagen type-IV mRNA expression was calculated from the ratio of collagen type IV to β2-microglobulin strength. To confirm the reproducibility of collagen type-IV/β2m gene expression, the RT-PCR of one synovial cell culture was repeated three times. Results showed a maximum variability of 15% (data not shown). Therefore, only differences of collagen type-IV expression of 15% or more were considered relevant.

Statistical analysis

Correlations between collagen type-IV staining and inflammation score/therapy were calculated using the rank correlation Spearman test.

Results

Inflammation score, clinical, and immunostaining data

Histologic scores of inflammation, immunohistochemical results, and patient data are listed in Table 1. Normal synovial membranes were histologically devoid of inflammatory changes, and the synovial lining layer was generally 1–3 cells in thickness. We found a strong positivity for collagen type IV both in all synovial layer cells and in the subintimal arterial and venular vessels (Fig. 1a, b).

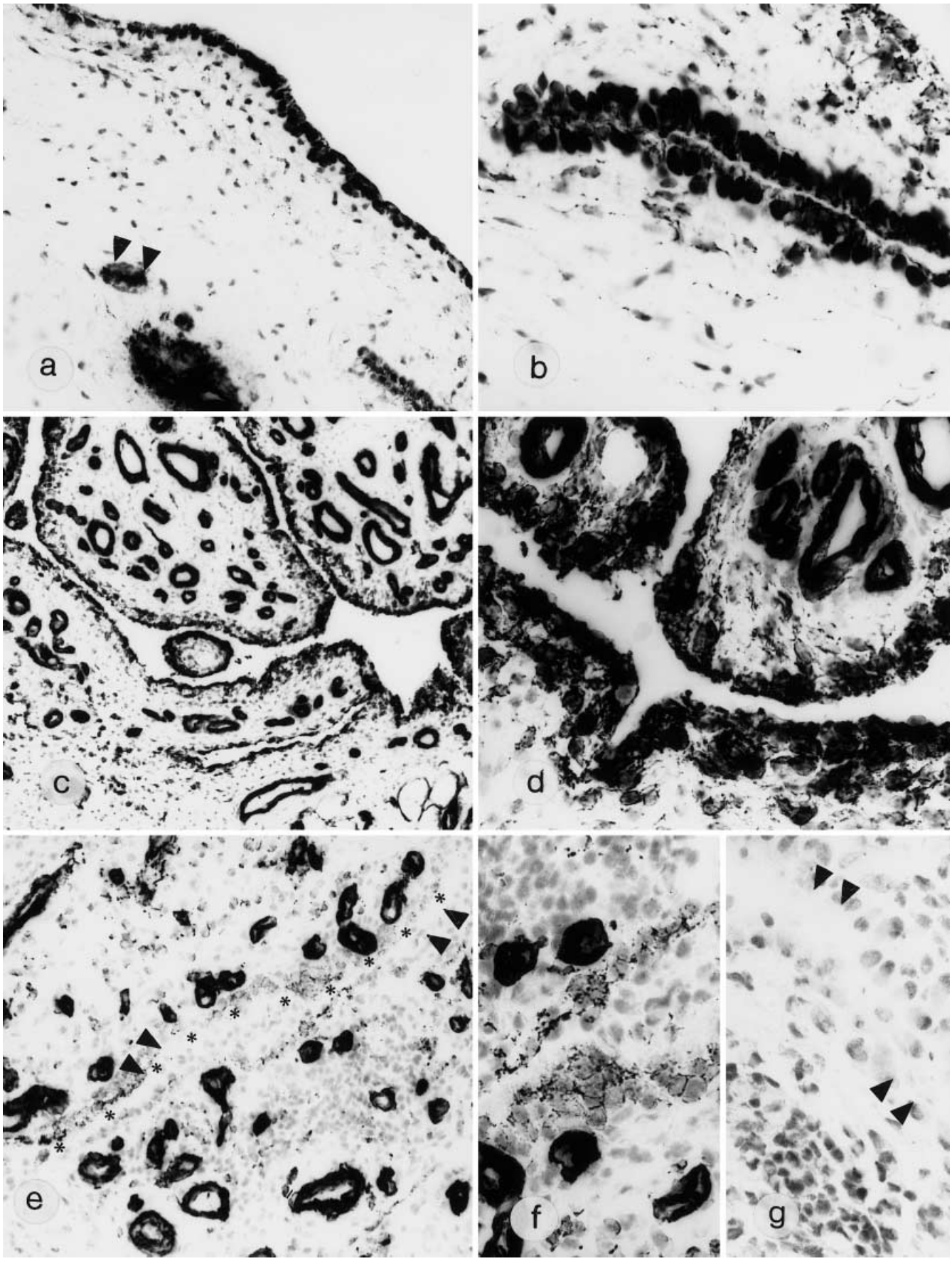
In OA synovia, the mean inflammation score was higher (mean±SD 6.2±2.1) than that of normal synovia. In only 1 of the 12 synovia samples was the inflammation score greater than 10. In 9 of 12 OA samples, SLCs were distributed in 1–3 cell layers, whereas in 3 of 12 OA samples the cell layer number was comprised within 4 and 5 cell layers. No correlation was found between the inflammation score and the synoviocyte hyperplasia. These parameters did not correlate with the laboratory test ESR. In 9 of 12 OA samples, the majority (samples 3, 6, 7, 8, 9, 10, and 14) or all (samples 5 and 12) SLCs showed collagen type-IV staining (Fig. 1c, d). In two samples (samples 4 and 11), a faint positivity for collagen type IV was distributed in 25–50% of SLCs. Only in one case (sample 13) was a weak collagen type-IV staining found in a low number of SLCs. Collagen type-IV staining did not correlate with the inflammation score or with the thickness of the synovial layer.

RA samples showed a high grade of inflammation (10.6±3.2). The degree of inflammation correlated statistically with the synovial lining hyperplasia. Collagen type-IV staining was heterogeneous in RA samples. Only in 3 of 19 RA samples did all (sample 18) or the majority (samples 28 and 33) of the SLCs show a collagen type-IV staining. In 6 (samples 15, 19, 21, 23, 25, and 26) and in 5 (16, 20, 24, 27, and 32) of 19 RA samples, collagen type-IV staining was found in 25–50% and in 1–25% of SLCs, respectively (Fig. 1e, f, g). The staining was mostly weak and distributed in the superficial synovial lining layers. In the other 5 RA samples (samples 17, 22, 29, 30, and 31), no collagen type-IV staining was found, whereas vessels were strongly collagen type-IV positive. The distribution of collagen type-IV staining inversely correlated with the degree of inflammation (P=0.002) and with the thickness of the synovial membrane (P=0.026).

Semi-quantitative RT-PCR of collagen type-IV gene expression

In order to investigate the factors playing a role in the synthesis of collagen type IV, RT-PCR analysis was performed in FLS isolated from synovia of patients with OA and RA. In pilot experiments, we established optimal reaction conditions for a semi-quantitative non-radioactive RT-PCR of collagen type-IV mRNA using a β2-microglobulin-specific PCR reaction as an internal control. In order to test the specificity of the PCR fragment, we blotted and hybridized the PCR products with digoxigenated oligonucleotide probes specific to collagen type IV and β2-microglobulin cDNA, which yielded one sharp, distinctive band of the expected size for each pair of primers (Fig. 3a). Since in situ results have shown differences in collagen type-IV staining related to the origin of the synovial membrane, we investigated the gene expression of collagen type IV in FLS from OA and RA. To determine whether the culture time influences the gene expression of collagen type IV, we established the RT-PCR analysis for FLS cultures after 1, 3, 5, and 8 weeks. Signal

Fig. 1 Detection of collagen type IV in normal synovia (**a**, **b**), osteoarthritis (**c**, **d**), and rheumatoid arthritis (**e**, **f**, **g**). Normal synovia (sample 1): (**a**, **b**) synovial lining cells (SLCs) are organized in a monolayer and show strong pericellular staining for collagen type IV; small vessels are strongly collagen type-IV positive (*arrowheads*). Osteoarthritis (sample 5): (**c**, **d**) SLCs form a pluricellular layer and a marked vascularization is noted. SLCs show the same strong collagen type-IV staining as the numerous intermingled small vessels. Rheumatoid arthritis (sample 23): (**e**) the synovia is hyperplastic with interspersed lymphoid aggregates. *Asterisks* mark the synovial cleft. Collagen type IV is detected only in a weak fashion in the synovial layers (*arrowheads*). At higher magnification, the hyperplastic SLCs show a weak and granular staining for collagen type IV (**f**) or are completely negative for collagen type IV (**g**); (original magnification **a**, **c**, **e**: ×71; **b**, **d**, **f**, **g**: ×140) ▶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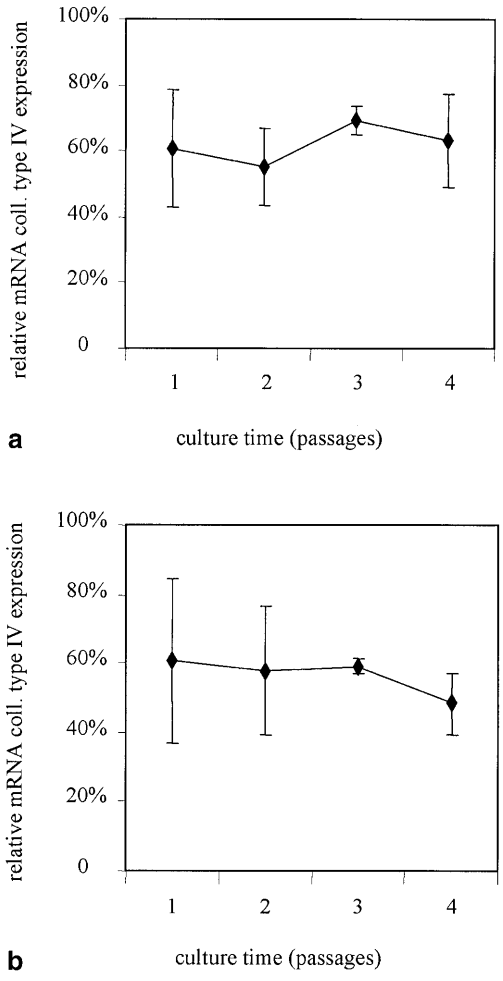

Fig. 2 Collagen type-IV mRNA expression of cultures of three fibroblast-like synoviocytes from osteoarthritis patients (OA-FLS; **a**) and three fibroblast-like synoviocytes from rheumatoid arthritis patients (RA-FLS; **b**) over a period of 6 weeks. mRNA expression is indicated as a mean value of the three cell cultures after normalization to β2-microglobulin signal. There are no relevant differences in the gene expression of collagen type IV between OA-FLS and RA-FLS at the indicated passages

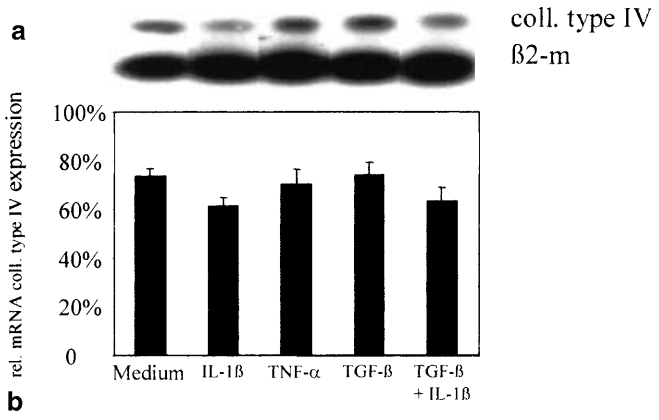

Fig. 3 mRNA collagen type-IV expression after cytokine stimulation in fibroblast-like synoviocytes from osteoarthritis patients (OA-FLS). **a** Southern-blot analysis of collagen type IV and β2-microglobulin mRNA expression in a representative experiment. No difference is seen in collagen type-IV expression relative to β2-microglobulin. **b** mRNA collagen type-IV expression is given as a mean value of three independent experiments after normalization to β2-microglobulin signal. Cytokines have no or only a minimal effect on the gene expression of collagen type IV in OA-FLS

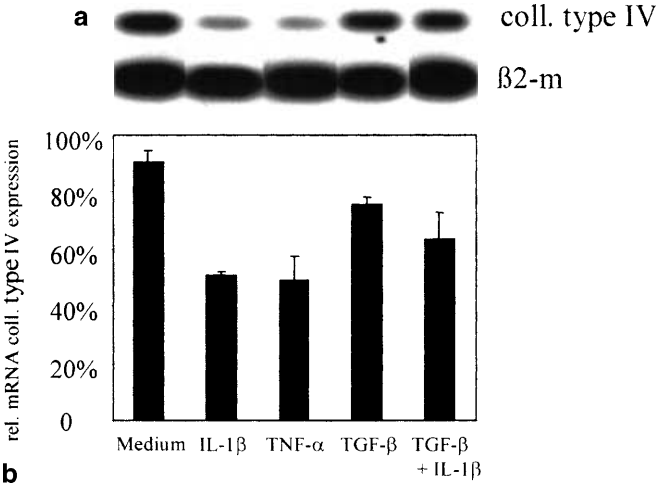

Fig. 4 mRNA collagen type IV expression after cytokine stimulation in fibroblast-like synoviocytes from rheumatoid arthritis patients (RA-FLS). **a** Southern-blot analysis of collagen type IV and β2-microglobulin mRNA expression in a representative experiment. The bands for collagen type IV are weaker for stimulation with interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and for transforming growth factor (TGF)-β plus IL-1β when compared with the control. **b** mRNA collagen type-IV expression is given as a mean value of three independent experiments after normalization to β2-microglobulin signal. IL-1β, TNF-α used alone or in combination show a downregulating effect on the gene expression of collagen type IV in RA-FLS, whereas no effect is shown after treating RA-FLS with TGF-β alone

strength of the collagen type-IV products obtained were compared with each other after normalization to the housekeeping gene β2-microglobulin.

As shown in Fig. 2a, b, we observed no relevant differences in the mRNA expression for collagen type IV between OA-FLS and RA-FLS. No differences in the collagen type-IV expression level were detectable between OA-FLS and RA-FLS, leading to the assumption of a comparable constitutional collagen type-IV mRNA expression.

OA-FLS and RA-FLS were treated with the cytokines IL-1β, TNF-α, and TGF-β, either alone or in combination to assess the influence on collagen type-IV gene expression. As shown in Fig. 3a, b, no relevant differences (=15%) were found for the gene expression of collagen type IV in cytokine-stimulated OA-FLS when compared with control cells. A relevant effect was shown by treatment of the RA-FLS with IL-1β and TNF-α (Fig. 4a, b). A decrease of collagen type-IV gene expression of circa 50% was found on adding IL-1β and TNF-α relative to the control (81.8%).

No synergistic effect was observed by adding IL-1β and TNF-α. Treatment of RA-FLS with TGF-β had no

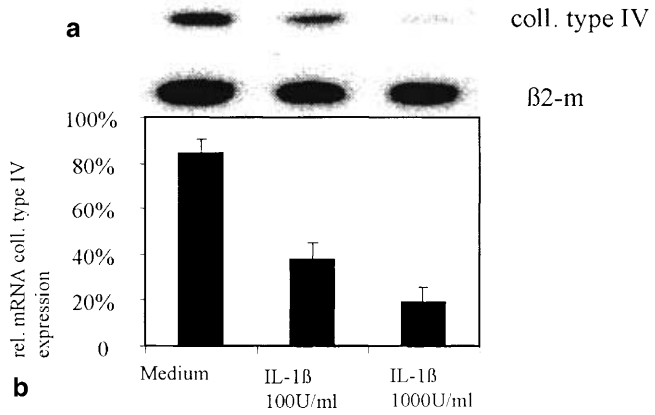

Fig. 5 mRNA collagen type-IV expression after stimulation in fibroblast-like synoviocytes from rheumatoid arthritis patients (RA-FLS) with various concentrations of interleukin (IL)-1β. **a** Southern-blot analysis of collagen type IV and β2-microglobulin mRNA expression. **b** A representative experiment shows weaker bands in correlation to the high concentration of IL-1β used. mRNA collagen type-IV expression is indicated as a mean value of three independent experiments after normalization to β2-microglobulin signal. Dose-dependent effect of IL-1β on the gene expression of collagen type IV is evident

relevant effect on the collagen type-IV gene expression (72.7%). However, the combination of IL-1β and TGF-β led to a decrease in collagen type-IV gene expression (63.9%). In the same FLS cultures stimulated with various concentrations of IL-1β, we showed that the effect on RA-FLS was dose dependent. As shown in Fig. 5, the original collagen type-IV gene expression (s. medium 85%) was downregulated by IL-1β used at a concentration of 100 U/ml to 38% and by IL-1β used at a concentration of 1000 U to 25% (Fig. 5a, b), whereas no relevant differences were observed in the collagen type-IV gene expression in OA-FLS by IL-1β used at higher concentrations.

Discussion

In agreement with other immunohistological studies, we observed that SLC in normal synovia in situ show a pericellular staining for the structural protein collagen type IV, suggesting that this protein may contribute to the structural organization of the synovial membrane [19, 24]. This study revealed a loss of collagen type IV in SLC of rheumatoid synovia. The decrease of collagen type IV in rheumatoid synovia correlated statistically with the grade of inflammation of the rheumatoid synovia and with the hyperplasia of the synovial cell layer. The loss of collagen type IV and of other basement membrane components may facilitate the infiltration of inflammatory cells in the synovial lining layer contributing to the pannus formation in the rheumatoid process [26, 31]. Previous in situ studies showed that collagen type IV is found surrounding the fibroblast-like synoviocytes in normal, OA and RA synovia [19, 29], suggesting that these cells may be responsible for the decrease of collagen type IV in rheumatoid arthritis.

This study revealed, that FLS in vitro, independent of origin, show a constitutive gene expression for collagen type IV. This finding is in agreement with immunofluorescence studies showing a collagen type-IV staining in cultured FLS [28]. In vitro, no differences in the baseline gene expression for collagen type IV were observed between RA-FLS and OA-FLS.

Since the presence of collagen type IV in rheumatoid synovia in situ correlated with the grade of inflammation and with the thickness of the synovial layer, we stimulated OA-FLS and RA-FLS with the cytokines involved in the inflammatory process in vivo [5, 10]. While TGF-β had no effect on the mRNA expression of collagen type IV, TNF-α and IL-1β downregulated the gene expression of collagen type IV in RA-FLS. The increase of the concentration of IL-1β led to a further downregulation of the gene expression of this protein. Since IL-1β is not only produced by inflammatory cells but also by synovial lining cells [13], this may explain the in situ findings, showing an inverse correlation between collagen type IV and the hyperplasia of the synovial lining cells. IL-1β and TNF-α are strong stimuli for the production of arachidonic acid metabolites, such as PGE2 in fibroblast-like synoviocytes [12]. Since PGE2 may decrease collagen synthesis, the downregulating effect by IL-1β and TNF-α on collagen type-IV gene expression may be mediated by PGE2 [20]. A downregulating effect on collagen gene expression was also observed in cultured human condrocytes by TNF-α and interferon (IFN)-γ [21]. However, IL-1β used at various concentrations showed a marginal effect on the gene expression of collagen type IV in OA-FLS. This suggests that the consistent presence of collagen type IV observed in SLC of OA-synovia may not only be due to a low grade of inflammation. The data suggest that the different regulation of the gene expression of collagen type IV by IL-1β in RA-FLS and OA-FLS may be caused by a different reactivity of these cells. The hypothesis of a different functional state of RA-FLS is supported by experimental evidence showing genetic mutations and different gene regulation in cultured RA-FLS [3, 7, 22, 32]. Previous data have shown enhanced serum concentrations of 7s-collagen, a marker of type-IV collagen metabolism, in patients with RA [11]. Therefore, apart from the direct effect of cytokines on the synthesis of collagen type IV, the presence of proteolytic enzymes for this matrix component like metalloproteinases may contribute to the degradation of this extracellular matrix protein [16].

In conclusion, the loss of collagen type IV in SLCs of rheumatoid synovia in situ correlates with the degree of inflammation and with the thickness of the synovial layer. Our in vitro results show that IL-1β and TNF-α have a downregulating effect on the gene expression of collagen type IV, suggesting that these cytokines may control the synthesis of collagen type IV in the rheumatoid process in vivo.

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References

1. Abrahamsen TG, Froland SS, Natvig JB, Pahle J (1975) Elution and characterization of lymphocytes from rheumatoid inflammatory tissue. Scand J Immunol 4:822–830
2. Adachi E, Hopkinson I, Hayashi T (1997) Basement-membrane stromal relationships: interactions between collagen fibrils and the lamina densa. Int Rev Cytol 173:73–156
3. Aicher WK, Heer AH, Trabandt A, Bridges SL, Schroeder HW, Stransky G, Gay RE, Eibel H, Peter HH, Siebenlist U (1994) Overexpression of zinc-finger transcription factor Z-225/Egr-1 in synoviocytes from rheumatoid arthritis patients. J Immunol 152:5940–5948
4. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, Christy W, Cooke TD, Greenwald R, Hochberg M, Howell D, Kaplan D, Koopman W, Longley S, Mankin H, McShane DJ, Medsger T, Meenan R, Mikkelsen W, Moskowitz R, Murphy W, Rothschild B, Segal M, Sokoloff L, Wolfe F (1988) Development of criteria for the classification and reporting of osteoarthritis: classification of osteoarthritis of the knee. Arthritis Rheum 29:1039–1049
5. Arend WP, Dayer JM (1990) Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis. Arthritis Rheum 33:305–315
6. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healy LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA, Mitchell DM, Neustadt DH, Pinals RS, Schaller Sharp JT, Wilder RL, Hunder GG (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315–324
7. Aupperle KR, Boyle DL, Hendrix M, Seftor EA, Zvaifler NJ, Barbosa M, Firestein GS (1998) Regulation of synoviocyte proliferation, apoptosis, and invasion by the p53 tumor suppression gene. Am J Pathol 152:1091–1098
8. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
9. Church MG, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci U S A 81:1991–1995
10. Feldmann M, Brennan FM, Chantry D, Haworth C, Turner M, Katsikis P, Londei M, Abney E, Buchan G, Barrett K (1991) Cytokine assays: role in evaluation of the pathogenesis of autoimmunity. Immunol Rev 119:105–123
11. Gabazza EC, Osamu T, Yamakami T, Ibata H, Sato T, Sato Y, Shima T (1994) Correlation between clotting and collagen metabolism markers in rheumatoid arthritis. Thromb Haemost 71: 199–202
12. Gitter BD, Labus JM, Lees SL, Scheetz ME (1989) Characteristics of human synovial fibroblast activation by IL-1 beta and TNF alpha. Immunology 66:196–200
13. Goto M, Okamoto M, Sasano M, Yanagisawa S, Miyamoto T, Nishioka K (1990) Adherent synovial cells from non-rheumatoid arthritis do not release interleukin-1 beta and prostaglandin E2 spontaneously in long term culture. J Rheumatol 17:1299–1302
14. Hostikka SL, Tryggvason K (1988) The complete primary structure of the alpha 2 chain of human type IV collagen and comparison with the alpha 1(IV) chain. J Biol Chem 263: 19488–19493
15. Khalkhali Ellis Z, Seftor EA, Nieva DR, Seftor RE, Samaha HA, Bultman L, De Larco JE, Ince A, Moore TL, Hendrix MJ (1997) Induction of invasive and degradative phenotype in normal synovial fibroblasts exposed to synovial fluid from patients with juvenile rheumatoid arthritis: role of mononuclear cell population. J Rheumatol 24:2451–2460
16. Martel Pelletier J, McCollum R, Fujimoto N, Obata K, Cloutier JM, Pelletier JP (1994) Excess of metalloproteases over tissue inhibitor of metalloproteinase may contribute to cartilage degradation in osteoarthritis and rheumatoid arthritis. Lab Invest 70:807–815
17. McCarthy JB, Vachhani B, Iida J (1996) Cell adhesion to collagenous matrices. Biopolymers 40:371–381
18. Okada Y, Morodomi T, Enghild JJ, Suzuki K, Yasui A, Nakanishi I, Salvesen G, Nagase H (1990) Matrix metalloproteinase 2 from human rheumatoid synovial fibroblasts. Purification and activation of the precursor and enzymic properties. Eur J Biochem 194:721–730
19. Pollock LE, Lalor P, Revell PA (1990) Type IV collagen and laminin in the synovial intimal layer: an immunohistochemical study. Rheumatol Int 9:277–280
20. Raisz LG, Pilbeam CC, Fall PM (1993) Prostaglandins: mechanisms of action and regulation of production in bone. Osteoporos Int 3[Suppl 1]:136–140
21. Reginato AM, Sanz-Rodriguez C, Diaz A, Dharmavaram RM, Jimenez SA (1993) Transcriptional modulation of cartilage-specific gene expression by interferon gamma and tumor necrosis factor alpha in cultured human condrocytes. Biochem J 294:761–769
22. Reme T, Travaglio A, Gueydon E, Adla L, Jorgensen C, Sany J (1998) Mutation of the p53 tumor suppressor gene in erosive rheumatoid synovial tissue. Clin Exp Immunol 111:353–358
23. Revell PA (1989) Synovial lining cells. Rheumatol Int 9:49–51
24. Revell PA, Al-Saffar N, Fish S, Osei D (1995) Extracellular matrix of the synovial intimal cell layer. Ann Rheum Dis 54: 404–407
25. Rinaldi N, Barth T, Henne C, Mechtersheimer G, Möller P (1994) Synoviocytes in chronic synovitis in situ and cytokine stimulated synovial cells in vitro neo-express α1, α3 and α5 chains of ß1 integrins. Virchows Arch 425:171–180
26. Rinaldi N, Barth TFE, Weis D, Schwarz-Eywill M, Pezzutto A, Lukoschek M, Brocai D, Brado B (1998) Loss of laminin and of the laminin receptor integrin subunit α6 in situ correlates with cytokine induced down-regulation of α6 on fibroblast-like synoviocytes from rheumatoid arthritis. Ann Rheum Dis 57:559–565
27. Rooney M, Condell D, Quinlan W, Daly L, Whelan A, Feighery C, Bresnihan B (1988) Analysis of the histologic variation of synovitis in rheumatoid arthritis. Arthritis Rheum 31: 956–963
28. Schneider M, Voss B, Rauterberg J, Menke M, Pauly T, Miehlke RK, Friemann J, Gerlach U (1994) Basement membrane proteins in synovial membrane: distribution in rheumatoid arthritis and synthesis by fibroblast-like cells. Clin Rheumatol 13:90–97
29. Smith SC, Folefac VA, Osei DK, Revell PA (1998) An immunocytochemical study of the distribution of proline-4-hydroxylase in normal, osteoarthritic and rheumatoid arthritic synovium at both the light and electron microscopic level. Br J Rheumatol 37:287–291
30. Soininen R, Haka-Risku T, Prockop DJ, Tryggvason K (1987) Complete primary structure of the alpha 1-chain of human basement membrane (type IV) collagen. FEBS Lett 225: 188–194
31. Suzuki M, Uetsuka K, Suzuki M, Shinozuka J, Nakayama H, Doi K (1997) Immunohistochemical study on type II collagen-induced arthritis in DBA/1J mice. Exp Anim 46:259–267
32. Trabandt A, Aicher WK, Gay RE, Sukhatme VP, Fassbender HG, Gay S (1992) Spontaneous expression of immediately-early response genes c-fos and egr-1 in collagenase-producing rheumatoid synovial fibroblasts. Rheumatol Int 12:53–59