Preferential Induction of Prodestructive Matrix Metalloproteinase-1 and Proinflammatory Interleukin 6 and Prostaglandin E_2 in Rheumatoid Arthritis Synovial Fibroblasts via Tumor Necrosis Factor Receptor-55

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ABSTRACT. ***Objective.*** To assess expression and individual functional relevance of tumor necrosis factor receptor 55 (TNF-R55) and TNF-R75 in rheumatoid arthritis (RA) and osteoarthritis (OA) synovial fibroblasts (SFB).

Methods. Seventh to 9th passage RA SFB and OA SFB were analyzed for TNF-R expression by FACS. The SFB were then stimulated with TNF-α (1–10 ng/ml) or agonistic anti-TNF-R55 (HTR-9) and anti-TNF-R75 (UTR-1) monoclonal antibodies (1–25 µg/ml each). Matrix metalloproteinase-1 (MMP-1), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), interleukin 6 (IL-6), and prostaglandin E_2 (PGE_2) in culture supernatants were quantified by ELISA, and DNA fragmentation by TUNEL assay.

Results. RA SFB variably expressed TNF-R55 (7.2 ± 2.2% positive cells, mean ± SEM) and TNF-R75 (0.6 ± 0.3%), similarly to OA SFB (6.8 ± 2.1% and 1.6 ± 0.8%, respectively). RA SFB constitutively secreted large amounts of TIMP-1 (1700 ng/ml), but only small amounts of MMP-1 (23.7 ng/ml), IL-6 (4.4 ng/ml), and PGE_2 (0.34 ng/ml). OA SFB secreted comparable amounts of TIMP-1 (2470 ng/ml), MMP-1 (37 ng/ml), and IL-6 (5.0 ng/ml), but significantly higher amounts of PGE_2 (0.58 ng/ml; $p \leq 0.05$). TNF-α stimulation induced IL-6 secretion by RA SFB (3-fold) and OA SFB (4-fold), as well as MMP-1 secretion (RA, 85-fold; OA, 29-fold), with significant differences between RA and OA. This was exclusively mediated by separate stimulation with agonistic anti-TNF-R55 Mab. Strikingly, RA SFB were completely unresponsive to TIMP-1 mRNA and protein induction by TNF-α, whereas TIMP-1 mRNA and/or protein in OA SFB was significantly upregulated by TNF-α (2-fold; $p \leq 0.05$, OA > RA) and by separate stimulation of both TNF receptors. TNF-α-induced PGE_2 release by RA SFB (92-fold) and OA SFB (56-fold) was mediated by both TNF receptors; however, predominantly by TNF-R55. DNA fragmentation was induced exclusively by high concentrations of anti-TNF-R55 Mab and only in RA SFB.

Conclusion. These results indicate preferential induction of prodestructive and proinflammatory mediators in RA SFB by the TNF-R55, with potential implications for understanding the pathogenesis of RA and the development of more specific therapeutic strategies. (J Rheumatol 2003;30:1680–90)

Key Indexing Terms:
TUMOR NECROSIS FACTOR-α AGONISTIC ANTIBODIES TNF-R55 TNF-R75 SYNOVIAL FIBROBLASTS

Activated synovial fibroblasts (SFB) contribute to the inflammatory and destructive potential of the aggressive pannus tissue in patients with rheumatoid arthritis (RA)[1-6] by producing soluble proinflammatory mediators [e.g., interleukin 6 (IL-6) and prostaglandin E_2 (PGE_2)][7,8] and matrix-degrading enzymes [e.g., matrix metalloproteinase-1 (MMP-1)][9,10].

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Tumor necrosis factor-α (TNF-α), a pleiotropic cytokine produced primarily by monocytes/macrophages, represents a major mediator of inflammatory, immunological, and pathophysiological reactions[11]. TNF-α is expressed as a bioactive 26 kDa precursor transmembrane molecule, and as a secreted mature 17 kDa cytokine, derived from the transmembrane form by proteolytic cleavage via TNF-α-converting enzyme (TACE/ADAM 17)[12]. The biological activity of TNF-α is mediated by binding to 2 distinct but related receptors, a protein of 55–60 kDa (TNF-R55) and another of 75–80 kDa (TNF-R75)[13,14].

TNF-α and its receptors play a major role in the pathogenesis of chronic inflammatory joint diseases such as human RA[15], as well as in several experimental arthritis models[16,17]. Most of the known cellular responses to TNF-α can be attributed to the activation of the TNF-R55, which initiates signals for cytotoxicity, fibroblast proliferation, cell adhesion to the endothelium, and induction of several genes[18-22]. TNF-R75, while sharing some activities with TNF-R55 and enhancing signaling of the latter via "ligand passing"[23], can in turn directly induce cellular responses independent of TNF-R55 stimulation and may play an accessory role in mediating other TNF effects, even if it is not responsible for the signal transmission[24-26].

In the RA synovial membrane, TNF-R55 and TNF-R75 are abundantly expressed in the lining layer[27], with a slight predominance of the latter[28]. In the sublining, fewer cells express TNF receptors, mostly of the TNF-R75 type. Diffuse infiltrates and lymphoid aggregates also contain 10–50% TNF-R75 positive cells, but only individual TNF-R55 positive cells[28]. While macrophages appear to be the predominant cells expressing TNF receptors, SFB (and T cells in lymphoid aggregates) also express TNF receptors, in the case of T cells exclusively TNF-R75[28].

To investigate the functional importance of the 2 TNF receptors for the proinflammatory and tissue-destructive potential of RA SFB, we analyzed the expression of the TNF receptors in SFB purified from primary culture[29] and subsequently passaged. Differential effects of TNF receptor stimulation on the production of MMP-1, tissue inhibitor of matrix metalloproteinases-1 (TIMP-1), IL-6, and PGE_2 and the induction of DNA fragmentation in RA SFB and OA SFB were analyzed using specific agonistic Mab against both TNF receptor types[22,30].

This study describes differential regulation of proinflammatory features of RA SFB and OA SFB by the 2 different TNF receptors, with potential implications for the development of innovative pathogenesis and treatment concepts for these diseases.

MATERIALS AND METHODS

Patients. Synovial tissues were obtained during open joint replacement surgery or arthroscopic synovectomy from 6 patients with the clinical diagnosis of RA and 5 with OA. Table 1 shows the clinical characteristics of all patients. All patients with RA fulfilled the American Rheumatism Association (ARA) criteria for RA[31]. The tissue was placed in cell culture medium at ambient temperature and subjected to tissue digestion within 2 h.

The study was approved by the ethics committees of the respective universities in Germany and the USA.

Tissue digestion and cell culture conditions. RA and OA synovial tissue samples were finely minced, then digested in phosphate buffered saline containing 0.1% trypsin (Sigma, Deisenhofen, Germany) and 0.1% collagenase P (Boehringer Mannheim, Mannheim, Germany) in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS), and subsequently cultured 7 days in DMEM/10% FCS, 25 mM HEPES, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml) (all Gibco BRL, Eggenstein, Germany), as described[29]. Samples were randomly tested to exclude *Mycoplasma* contamination (Mycoplasma detection kit; Boehringer Mannheim).

Negative isolation of SFB from primary cultures of synovial cells. SFB were negatively isolated from trypsinized RA and OA synovial primary culture cells (passage 0) using Dynabeads® M-450 CD14 (clone RMO52; Dynal, Hamburg, Germany), as described[29]. SFB were > 89% prolyl-4-hydroxylase+; > 75% Thy-1+ (CD90+); < 2% CD14+ and CD68+; < 1% CD3+, CD19+/20+, CD38+, CD56+, CD83+, CD15+, CD144+, von Willebrand factor+[29], i.e., after isolation from primary culture, SFB were cultured in the virtual absence of contaminating nonadherent cells and macrophages.

Flow cytometry. Seventh to 9th passage RA SFB and OA SFB were analyzed by FACS for surface expression of TNF-α, TNF receptors, and Fas using the antibodies listed in Table 2. Primary antibodies were used at concentrations of 10–20 µg/ml. After trypsinization, 2×10^5 SFB were suspended in 100 µl PBS/1% FCS/0.02% NaN_3. Unconjugated primary Mab were added for 30 min at 4°C, followed by incubation with FITC-labeled goat anti-mouse antibodies for 30 min at 4°C. The specificity of staining was confirmed using isotype matched control Mab at identical concentrations (Table 2). Analyses were performed on a FACS-Calibur® using the Cell Quest software (Becton Dickinson, San Jose, CA, USA). Forward/side scatter gates were set to include all viable cells. To determine the percentage of cells positive for each marker, a gate was placed at the intercept of the curves obtained with specific Mab and control immunoglobulins (Ig); the percentage of cells stained with control Ig was then subtracted from the percentage of cells stained with the specific Mab.

Stimulation of SFB with TNF-α or specific agonistic anti-TNF-R55 (HTR-9) or anti-TNF-R75 Mab (UTR-1). Recombinant human TNF-α (specific activity 7.69×10^7 U/ml; endotoxin content < 0.1 ng/mg cytokine, LAL assay) was purchased from Genenzyme (Haverford, PA, USA). The agonistic anti-TNF-R55 (HTR-9, IgG1) and anti-TNF-R75 Mab (UTR-1, IgG1; both kindly provided by Dr. M. Brockhaus, F. Hoffmann La Roche Ltd., Basel, Switzerland) were purified from hybridoma cell culture medium by affinity chromatography on anti-mouse Ig sepharose. Purity was roughly 95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

These antibodies were raised against partially purified preparations of TNF binding proteins isolated from HL-60 cells (HTR-9) or U937 cells (UTR-1) as described[30]. The combination of the Mab HTR-9 and UTR-1 fully inhibits the TNF binding to HL-60 and U937, which express both TNF receptors[30]. Specificity of the Mab was confirmed by flow cytometry, Western blot, and immunoprecipitation as described[30]. The HTR-9 Mab has agonistic effects in most TNF-R55 (CD120a) mediated signaling systems[32-34], whereas the UTR-1 Mab has agonistic properties in TNF-R75 (CD120b) mediated signaling[24]. Depending on the functional effects analyzed, the UTR-1 Mab can be partially inhibitory when low TNF-α concentrations (e.g., 1 ng/ml) are present in the culture system, probably because of the reduction in the overall affinity for TNF-α in cells expressing both receptor types[32,34-36], and presumably reflecting blockade of "ligand passing"[23].

RA SFB or OA SFB (7th–9th passage, 2×10^5 cells/well; n = 3 each for determination of IL-6, PGE_2, MMP-1, and TIMP-1) were maintained in 12 well plates (Costar, Buckinghamshire, UK) for 12 h prior to stimulation. After exchange of the medium (500 µl of DMEM/5% FCS), the cells were

Table 1. Clinical characteristics of patients with RA/OA at the time of synovectomy.

	Sex/Age	Disease Duration, yrs	RF	ESR, 1 h	CRP, mg/l	ARA Criteria for RA, n	Assay	Concomitant Medication
RA								
EB4	F 68	10	+	ND	84.7	5	T	NSAID, Pred
EB6	F 77	10	–	88	68.3	5	T	NSAID
EB7	M 48	2	+	76	135.9	6	F/A/T	NSAID, MTX, Pred, SSZ
EB26	M 60	ND	ND	ND	68.6	4	F/A	NSAID, Pred, SSZ
EB34	M 58	3	+	22	10.1	4	F/A	NSAID, leflunomide, Pred
EB52	M 76	5	+	45	38.5	6	F/A	NSAID
OA								
EB12	F 79	ND	ND	14	< 5	1	T	NSAID
EB13	M 72	ND	ND	2	< 5	0	F/A/T	None
EB14	F 68	ND	ND	ND	< 5	0	T	NSAID
EB54	F 76	4	ND	26	< 5	0	F/A	None
J4	F 65	10	ND	6	7.7	0	F/A	NSAID, Pred

RF: rheumatoid factor, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein (normal range < 5 mg/l), F: flow cytometry, A: apoptosis assay; T: TNF receptor Mab stimulation, ND: not determined, NSAID: nonsteroidal antiinflammatory drugs, MTX: methotrexate, Pred: prednisolone, SSZ: sulfasalazine.

Table 2. Antibodies used in the study.

Antibodies	Antigen Recognized	Cellular Localization	Main Cellular Expression/Ig Control	Assay	Source
MOPC 21			IgG1 isotype control	F/A/T	Sigma, Deisenhofen*
UPC-10			IgG2a isotype control	F	Sigma
Mouse IgM			IgM isotype control	F/A	Immunotech, Hamburg*
TIA2	TNF-α	Transmembrane/soluble	Macrophages, fibroblasts, T cells	F	Dr. H. Böttinger, Univ. of Stuttgart*
MAK195F	TNF-α	Soluble	Macrophages, fibroblasts, T cells	F	Dr. M. Kaul, Knoll AG, Ludwigshafen*
H398	TNF-R55 (CD120a)	Membrane	Macrophages, fibroblasts, T cells	F	Dr. M. Grell, Inst. of Cell Biology and Immunology, Univ. of Stuttgart*
80M2	TNF-R75 (CD120b)	Membrane	Macrophages, fibroblasts, T cells	F	Dr. M. Grell
HTR-9	TNF-R55	Membrane	Macrophages, fibroblasts, T cells	A/T	Dr. M. Brockhaus, F. Hoffmann La Roche Ltd., Basel, Switzerland
UTR-1	TNF-R75	Membrane	Macrophages, fibroblasts, T cells	A/T	Dr. M. Brockhaus
CH11	Fas (CD95)	Membrane	Wide variety of cells	F/A	Immunotech

F: flow cytometry, A: apoptosis assay, T: TNF receptor Mab stimulation. * Germany.

stimulated in duplicate cultures either without stimulus or with: (a) 1, 10, and 25 ng/ml of TNF-α; (b) 1, 10, and 25 µg/ml HTR-9 (agonistic anti-TNF-R55 Mab); or (c) 1, 10, and 25 µg/ml UTR-1 (agonistic anti-TNF-R75 Mab) for 30 h at 37°C in 5% CO_2. At the end of the incubation time, supernatants were collected from the cultures and kept frozen at –80°C until analysis was performed. The endotoxin content of the cell culture supernatants after addition of recombinant TNF-α or the TNF receptor Mab was always < 0.03 U/ml (LAL assay, Sigma). The use of murine IgG1 (negative control; 1, 10, and 25 µg/ml) in selected experiments yielded results comparable to those of medium only, the negative control routinely employed in this study (data not shown).

Measurement of human MMP-1 and TIMP-1 by sandwich ELISA. This assay (sensitivity 1.70 ng/ml for MMP-1 and 1.25 ng/ml for TIMP-1) was based on a 2-site ELISA sandwich format and was carried out according to the manufacturer's instructions (Biotrak™, Amersham-Pharmacia Biotech, Piscataway, NJ, USA). In the case of MMP-1, the assay recognizes total human MMP-1, i.e., free and TIMP-1 complexed MMP-1 (both the pro-form and the processed MMP-1), without crossreactivity to MMP-2, 3, and 9. In the case of TIMP-1, the assay recognizes total human TIMP-1, i.e., free and TIMP-1 complexed with MMP-1, 2, 3, 9, and pro-MMP-9, without crossreactivity to TIMP-2. The resulting color was read at 450 nm in a microtiter plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The concentrations of MMP-1 or TIMP-1 in diluted cell culture supernatants were determined by interpolation from a standard curve (range 6.25 to 100 ng/ml for MMP-1 and range 3.13 to 50 ng/ml for TIMP-1). Due to the absence of a secondary anti-mouse antibody, interference of the TNF receptor Mab with the assays was excluded (as also shown by the IgG1 control).

Extraction of RNA, reverse transcription, and RT-PCR for TIMP-1. Total cellular RNA was extracted from 2 × 10^5 cultured SFB using TRI Reagent™ (Sigma). Between 2 and 5 µg of total RNA were primed with Oligo(d)T and reverse-transcribed with SuperScript-II reverse transcriptase according to the manufacturer's instructions (Gibco-BRL).

TIMP-1 mRNA expression was quantified by 27 cycles of reverse transcription-polymerase chain reaction (downstream primer: ATCCTGTTGTTGCTGTGGCTGATAG; upstream primer: TGCTGGGTGGTAACCTCTTTATTTCA; resulting fragment length 667 bp): initial denaturation at 94°C for 30 s, subsequently cycles of denaturation at 94°C for 20 min, followed by annealing at 58°C for 1 min and extension at 72°C for 1 min 50 s. To normalize the samples for equal amounts of cDNA, the housekeeping gene aldolase (downstream primer: TCATCCTCTTCCATGAGACACTCTA; upstream primer: ATTCTGCTGGCAGATACTGGCATAA; resulting fragment length 314 bp) was used as an internal standard (initial denaturation at 94°C for 30 s, followed by cycles of denaturation at 94°C for 20 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min 50 s; total of 34 cycles). All primers were kindly provided by Dr. E. Birch-

Hirschfeld, Institute of Virology, Friedrich Schiller University, Jena, Germany. Product specificity was confirmed by fluorescent cycle sequencing of the PCR products.

Amplified products were separated and visualized in a 2% agarose gel (Invitrogen) containing 0.1 µg/ml ethidium bromide (Roth) at 280 nm. The intensity of each band was analyzed using integration image software (Scion Corp., Frederick, MD, USA).

Measurements of human IL-6. Human IL-6 was measured in diluted cell culture supernatants using a quantitative sandwich enzyme immunoassay technique according to the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, MN, USA; sensitivity 0.70 pg/ml). A wavelength of 450 nm with wavelength correction at 540 nm was employed to quantify the color reaction. Sample concentrations of IL-6 were determined by comparison with a standard curve (range 3.13 to 300 pg/ml). Due to the absence of a secondary anti-mouse antibody, interference of the TNF receptor Mab with the assay was excluded.

Measurement of PGE_2. This competition assay (sensitivity 36.2 pg/ml of PGE_2) was carried out according to the manufacturer's instructions (R&D Systems). The optical density in each well was determined at 405 nm with wavelength correction at 570 nm. The concentration of PGE_2 was determined by comparison with a standard curve (range 39 to 5000 pg/ml).

Assessment of DNA fragmentation (TUNEL assay). Passaged RA SFB (n = 4 experiments) and OA SFB (n = 3 experiments) were cultured either without stimulus or with: (a) 1, 10, and 25 ng/ml of TNF-α; (b) 1, 10, and 25 µg/ml HTR-9 (agonistic anti-TNF-R55 Mab); (c) 1, 10, and 25 µg/ml UTR-1 (agonistic anti-TNF-R75 Mab); (d) the combination of 100 ng/ml CH-11 Mab (agonistic anti-Fas/CD95 Mab of the IgM isotype; Immunotech, Hamburg, Germany) and cycloheximide (CHX; 10 µg/ml; Sigma) as a positive control for 30 h at 37°C in 5% CO_2. IgG1 or IgM in matched concentrations were used as negative controls. After stimulation, the percentage of cells positive for DNA strand breaks was determined using the TUNEL label nucleotide kit according to the manufacturer's guidelines (Boehringer Mannheim) and subsequent FACS analysis.

Statistical analysis. The nonparametric Mann-Whitney U test was applied for analysis of the phenotypic and functional features, with statistically significant differences accepted for $p \leq 0.05$. The Spearman rank correlation test was used to analyze correlations among experimental variables and between these variables and the clinical status/treatment of individual patients ($p \leq 0.05$). Analyses were performed using the SPSS 9.0 program (SPSS Inc., Chicago, IL, USA).

RESULTS

Expression of TNF-R55 and TNF-R75 on SFB. Variable percentages of RA SFB expressed TNF-R55 (7.2 ± 2.2%, mean ± SEM) and TNF-R75 (0.6 ± 0.3%; Figures 1 and 2). OA SFB expressed TNF-R55 (6.8 ± 2.1%) and TNF-R75 (1.6 ± 0.8%) comparably to RA SFB. The validity of these results (in particular the low percentages of TNF-R75+ cells) was confirmed by numeric and/or significant differences between the mean fluorescence indices of control IgG and the respective anti-TNF receptor Mab.

Neither transmembrane nor soluble, receptor-bound TNF-α was detected on RA SFB or OA SFB (mean percentages < 0.4% in both cases; Figures 1 and 2). The Fas molecule (CD95) was present on a high percentage of RA SFB (67.2 ± 7.1%) and OA SFB (57.8 ± 10.7%; Figure 1). None of the differences between RA and OA was statistically significant.

A significant, positive correlation, in turn, was observed between the expression of TNF-R55 and Fas in RA SFB and OA SFB (rho = 0.893, p = 0.007, n = 7, Spearman rank correlation).

Functional effects of agonistic anti-TNF-R55 or anti-TNF-R75 Mab on SFB. MMP-1: RA SFB and OA SFB (n = 3 each) constitutively secreted considerable amounts of MMP-1 (RA, 23.7 ng/ml, 0.0004 nmol/ml; OA, 37 ng/ml, 0.0007 nmol/ml; Figure 3), with no significant differences between RA and OA SFB. Incubation with TNF-α significantly stimulated MMP-1 secretion in both RA SFB (85-fold; 1899.7 ng/ml, 0.034 nmol/ml) and OA SFB (29-fold; 1085.0 ng/ml; 0.020 nmol/ml). This was exclusively mediated by TNF-R55 (i.e., agonistic anti-TNF-R55 Mab; Figure 3). RA SFB stimulated with TNF-α (1 ng/ml) secreted significantly more MMP-1 than OA SFB (Figure 3).

TIMP-1: Nonstimulated RA SFB and OA SFB (n = 3 each) secreted large amounts of TIMP-1 (RA, 1700 ng/ml, 0.06 nmol/ml; OA, 2470 ng/ml, 0.10 nmol/ml, respectively; Figure 3), with no significant differences between RA and OA. Strikingly, RA SFB were completely unresponsive to TIMP-1 induction by TNF-α stimulation, while in OA SFB TIMP-1 was significantly upregulated by TNF-α (2-fold; 5015.0 ng/ml, 0.197 nmol/ml). This effect was mediated by stimulation of both TNF-R55 and TNF-R75 (Figure 3). OA SFB stimulated with TNF-α (10 ng/ml or 25 ng/ml) or with agonistic anti-TNF receptor Mab (at all concentrations used) secreted significantly more TIMP-1 than RA SFB (Figure 3).

This differential induction of TIMP-1 by TNF-α in OA SFB and RA SFB was also confirmed at the mRNA level. In OA SFB (n = 3), TIMP-1 mRNA expression was induced by TNF-α (1.8-fold by 1 and 10 ng/ml TNF-α), agonistic anti-TNF-R55 Mab (2.3-fold and 1.2-fold, respectively, by 10 and 25 µg/ml), and anti-TNF-R75 Mab (1.3-fold and 2-fold, respectively, by 1 and 10 µg/ml; in the latter case $p \leq 0.05$ vs nonstimulated cells). RA SFB (n = 2), in contrast, showed no TIMP-1 mRNA upregulation in response to stimulation with TNF-α, anti-TNF-R55 Mab, or TNF-R75 Mab (at all concentrations used; data not shown).

The 85-fold induction of MMP-1 protein, as well as the lack of TIMP-1 protein induction by TNF-α in RA SFB, resulted in a marked increase in the MMP-1/TIMP-1 molar ratio (from 0.01 to 0.53) for RA, but a much smaller increase for OA (from 0.01 to 0.13; Figure 3). Accordingly, TNF-α-stimulated RA SFB (at all concentrations used) and anti-TNF-R55-stimulated RA SFB (25 µg/ml) showed a significantly higher MMP-1/TIMP-1 ratio than OA SFB (Figure 3).

IL-6: RA SFB and OA SFB (n = 3 each) constitutively expressed considerable amounts of IL-6 (4.4 and 5.0 ng/ml, respectively; Figure 4), with no significant differences between RA and OA. TNF-α incubation significantly stimulated IL-6 secretion in both RA SFB (3-fold; 13.0 ng/ml) and OA SFB (4-fold; 20.7 ng/ml), an effect exclusively mediated by TNF-R55 (i.e., agonistic anti-TNF-R55 Mab). Interestingly, stimulation of OA SFB with 1 or 10 ng/ml TNF-α resulted in significantly higher IL-6 secretion than stimulation of RA SFB (Figure 4).

PGE_2: Nonstimulated RA SFB and OA SFB (n = 3 each)

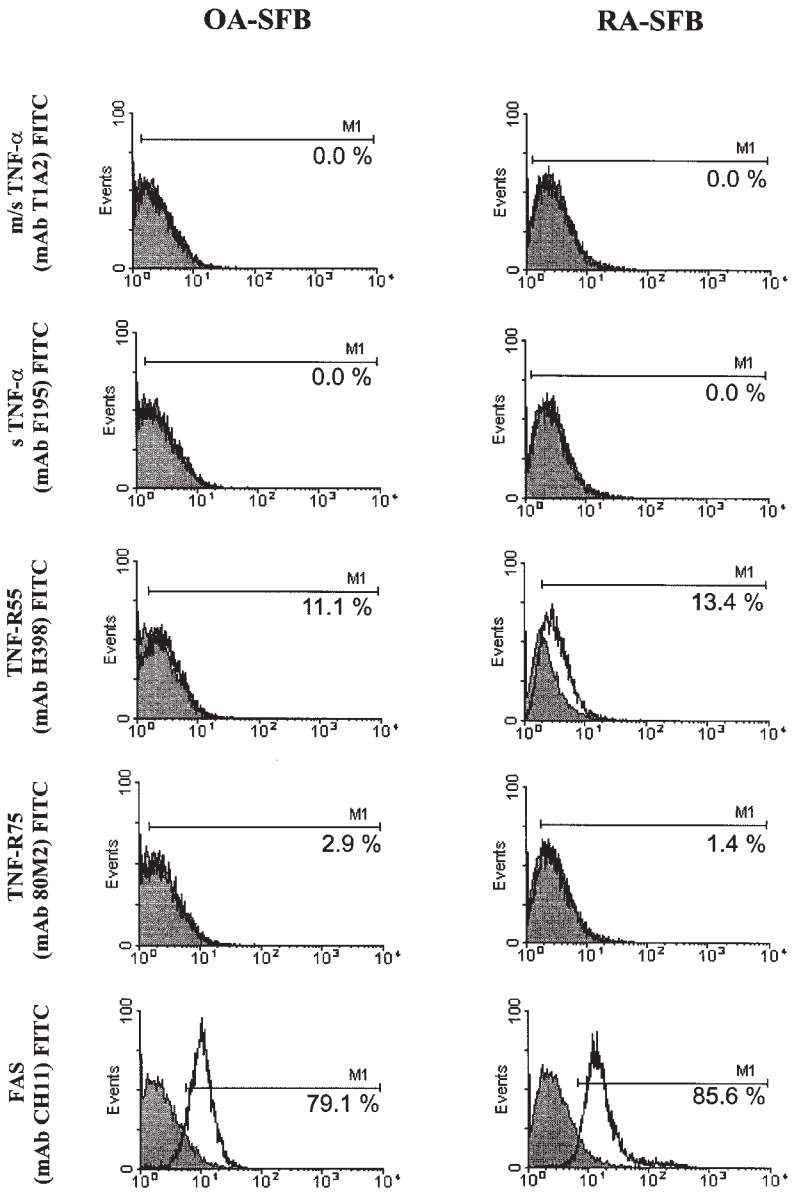

Figure 1. Expression of TNF-α, TNF-R55, TNF-R75, and Fas on passaged SFB from representative OA and RA patients (FACS analysis). OA and RA SFB did not show positive reaction for transmembrane or soluble, receptor-bound TNF-α, but a considerable percentage of the cells expressed TNF-R55 and TNF-R75. A large percentage of both OA and RA SFB expressed the Fas molecule.

secreted small amounts of PGE_2 (0.34 and 0.58 ng/ml, respectively; Figure 4), with significantly higher amounts in OA SFB. TNF-α incubation significantly stimulated PGE_2 secretion in both RA SFB (92-fold; 31.2 ng/ml) and OA SFB (56-fold; 32.7 ng/ml). This stimulation was mediated by both TNF receptors, however, with a predominance for TNF-R55. OA SFB stimulated with agonistic anti-TNF-R75 Mab (1 or 10 µg/ml) secreted significantly more PGE_2 than RA SFB (Figure 4).

DNA fragmentation: In order to rule out that the above noted functional effects, in particular the differential unresponsiveness of RA SFB to TNF-α-induced increase of TIMP-1 production, were partially or completely due to apoptosis, DNA fragmentation was assessed.

In RA SFB, significant DNA fragmentation in comparison to controls was induced only by high concentrations (25 µg/ml) of the agonistic anti-TNF-R55 Mab HTR-9 or by the combination of the anti-Fas Mab CH-11 with cycloheximide (CHX; positive control; Figure 5). The percentage of cells with DNA fragmentation after anti-TNF-R55 Mab

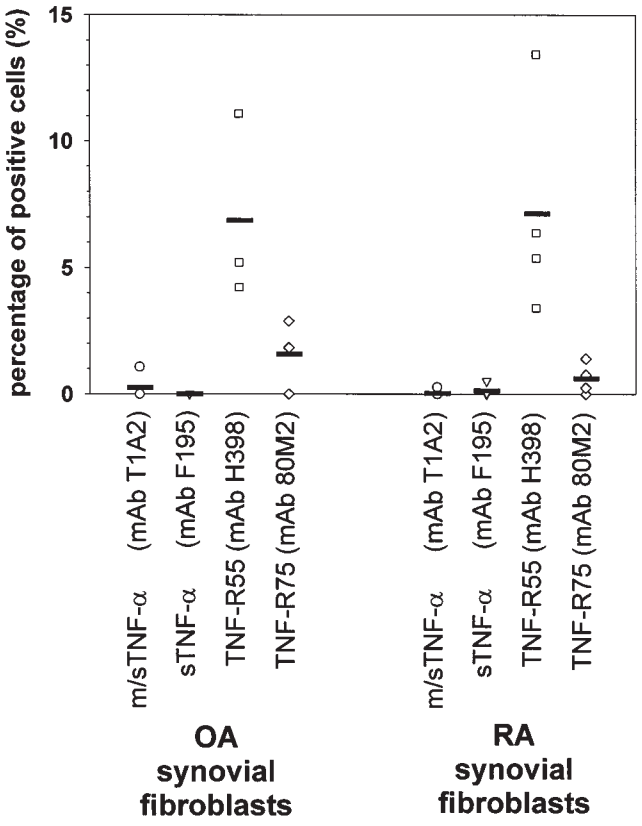

Figure 2. Expression of TNF-α, TNF-R55, and TNF-R75 on 7th to 9th passage SFB from OA (n = 3) and RA (n = 4) patients (FACS analysis). OA and RA SFB did not show a positive reaction for transmembrane or soluble, receptor-bound TNF-α (average of < 0.4% positive cells), but a considerable percentage of the cells expressed TNF-R55 (OA, average of 6.8%; RA, 7.2%) and TNF-R75 (OA, 1.6%; RA, 0.6%). There was no significant difference between OA and RA for any variable.

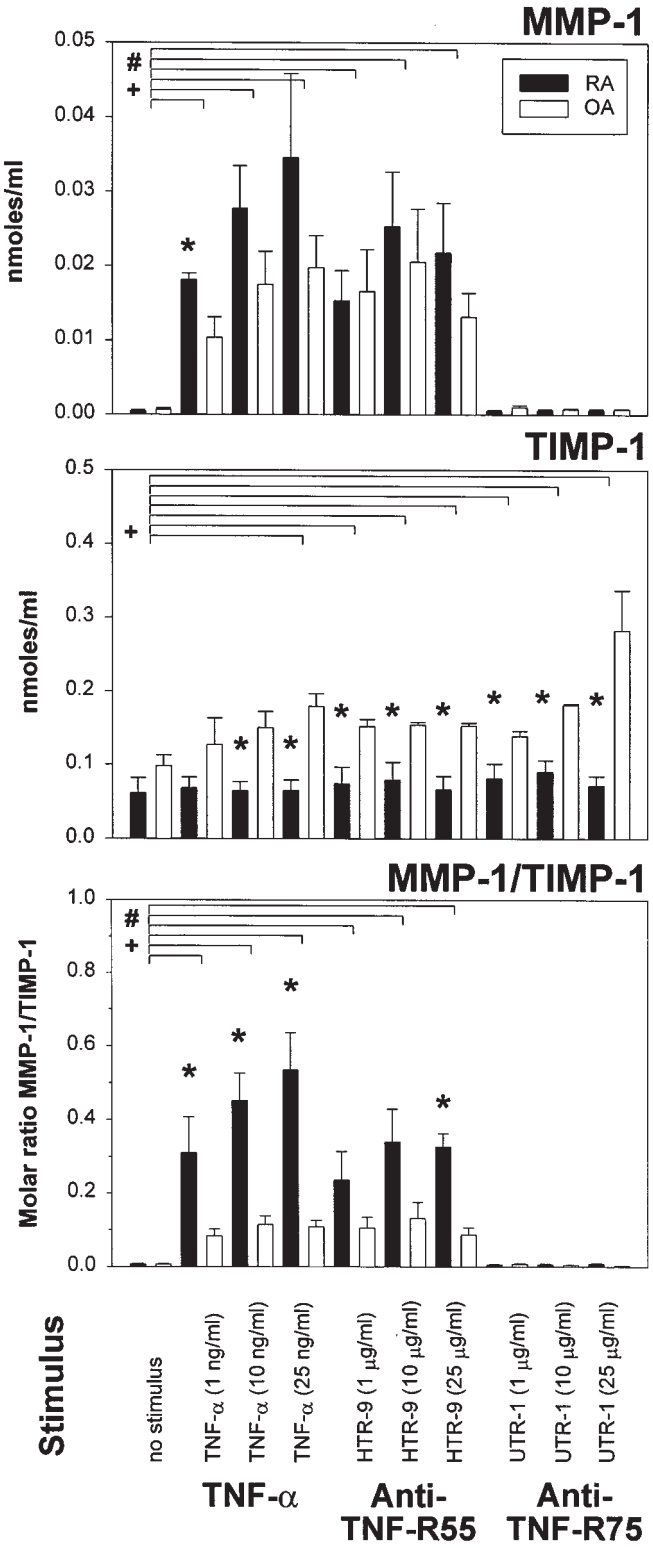

Figure 3. Differential regulation of MMP-1 and TIMP-1 in 7th to 9th passage SFB from OA and RA patients (n = 3 each) following stimulation with TNF-α or agonistic anti-TNF-R55 or anti-TNF-R75 Mab. The inductive effects of TNF-α on MMP-1 secretion in both OA and RA SFB were exclusively mediated by the TNF-R55. In contrast, induction of TIMP-1 secretion in OA SFB was equally mediated by both TNF receptors. Strikingly, RA SFB were completely unresponsive to TIMP-1 induction by TNF-α, resulting in significantly lower TIMP-1 levels than in OA SFB for almost all concentrations of TNF-α or agonistic anti-TNF receptor Mab. The 85-fold induction of MMP-1 and the lack of TIMP-1 induction by TNF-α in RA SFB resulted in an enormous increase in the MMP-1/TIMP-1 ratio (from 0.01 to 0.53) for RA, but a much smaller increase for OA (from 0.01 to 0.13). Accordingly, TNF-α-stimulated RA SFB (at all concentrations) and TNF-R55-stimulated RA SFB (25 μg/ml) showed a significantly higher MMP-1/TIMP-1 ratio than OA SFB. $^{\#}p \leq 0.05$ for comparison between nonstimulated controls and TNF-α or TNF receptor-stimulated RA SFB; $^{+}p \leq 0.05$ for comparison between nonstimulated controls and TNF-α or TNF receptor-stimulated OA SFB; $^{*}p < 0.05$ for comparison between OA and RA SFB.

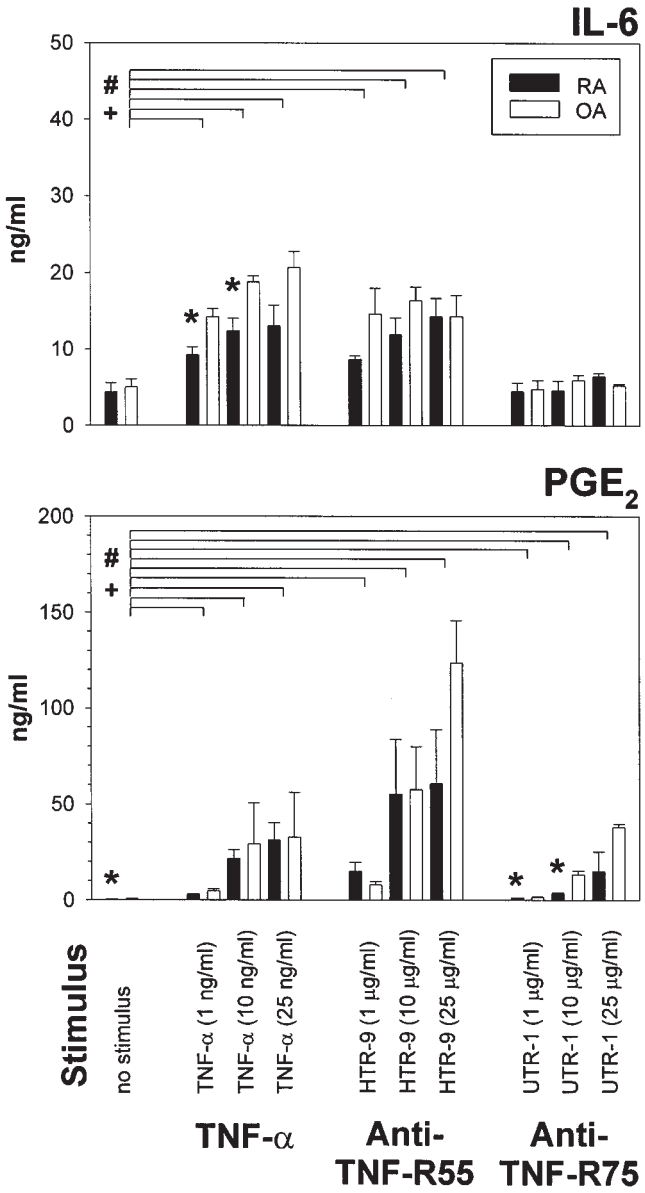

Figure 4. Differential regulation of IL-6 and PGE_2 in 7th to 9th passage SFB from OA and RA patients (n = 3 each) after stimulation with TNF-α or agonistic anti-TNF-R55 or anti-TNF-R75 Mab. While the inductive effects of TNF-α on IL-6 secretion in both OA and RA SFB were exclusively mediated by TNF-R55, effects of TNF-α on PGE_2 secretion were mediated by both TNF receptors, with a predominance for TNF-R55. $^{\#}p \leq 0.05$ for comparison between nonstimulated controls and TNF-α or TNF receptor-stimulated RA SFB; $^{+}p \leq 0.05$ for comparison between nonstimulated controls and TNF-α or TNF receptor-stimulated OA SFB; $^{*}p < 0.05$ for comparison between OA and RA SFB.

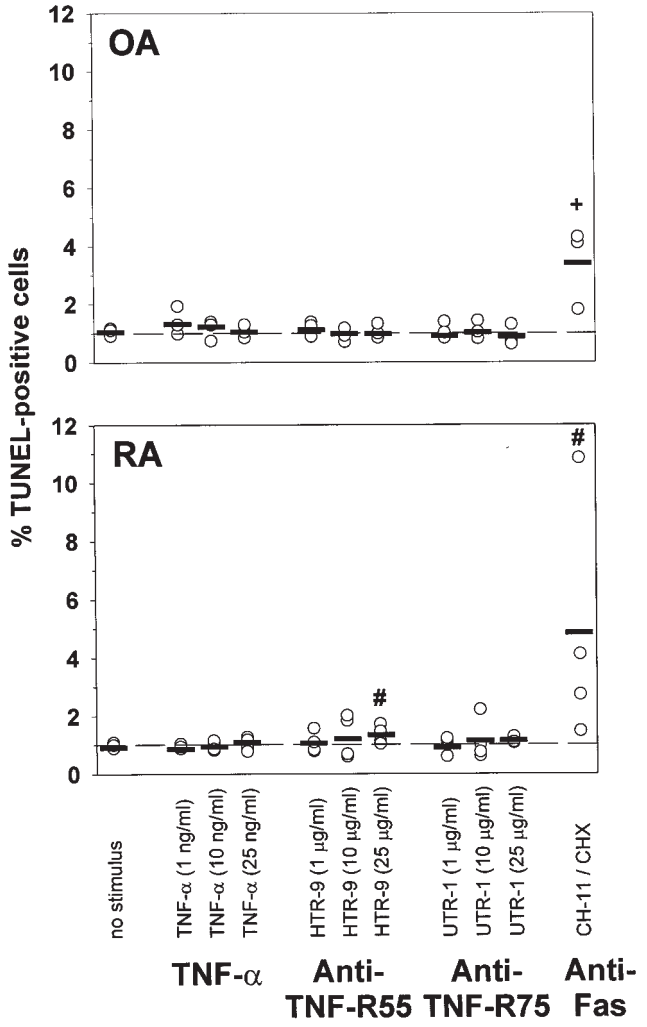

Figure 5. Assessment of DNA fragmentation in 7th to 9th passage SFB from OA (n = 3) and RA patients (n = 4) after stimulation with TNF-α or agonistic anti-TNF-R55, anti-TNF-R75, or anti-Fas Mab (TUNEL assay). In RA SFB, significant DNA fragmentation was only induced by the agonistic anti-TNF-R55 Mab HTR-9 (only at 25 μg/ml) and the combination of the anti-Fas Mab CH11 (100 ng/ml) and cycloheximide (CHX). In OA SFB, only the combination anti-Fas Mab CH11/CHX led to a significant increase of DNA fragmentation. $^{\#}p \leq 0.05$ for comparison with nonstimulated RA SFB; $^{+}p \leq 0.05$ for comparison with nonstimulated OA SFB; there were no significant differences between OA and RA for any variable. In both cases, there was no significant difference between nonstimulated cells and those stimulated with the same concentration of the respective isotype-matched immunoglobulin.

exposure was very low (roughly 1.3%), whereas a substantial percentage of RA SFB (roughly 4.8%) showed DNA fragmentation after stimulation with anti-Fas/CHX, in agreement with previous reports[37,38]. In OA SFB, only the combination of anti-Fas/CHX led to a significant increase of DNA fragmentation above control results (Figure 5). The results in RA and OA SFB did not differ significantly.

DISCUSSION

This study shows the presence of both TNF receptor types on the surface of rheumatoid and osteoarthritis synovial fibroblasts (Figures 1 and 2)[35,39,40], confirming findings in *ex vivo* synovial tissue and mixed synovial cells from primary culture (data not shown)[27,28,29,41]. In the case of RA SFB, the ratio between TNF-R55 and TNF-R75 was higher than previously observed (roughly 12 vs 3–4)[35]. This is conceivably due to variations of the clinical variables in the patient popu-

lations analyzed, and/or to the use of different analytical techniques (FACS analysis vs radioligand binding studies).

The percentage of SFB showing positive staining for the different TNF receptors in passage 0 was higher (OA SFB, 32.7% TNF-R55, 16.8% TNF-R75; RA-SFB, 46.0% TNF-R55, 20.4% TNF-R75; $p \leq 0.05$ for both TNF receptors in the case of RA SFB) than in passages 7–9 (Figures 1 and 2). Since passage 0 SFB have not been investigated to date and since all studies with passaged SFB have been performed with radioligands (not yielding the percentage of positive cells)[35,39,40], a comparison with literature reports appears difficult. This finding supports the view that receptor expression diminishes with repeated passages, and that the functional effects observed in this study may be even more pronounced with early-passage SFB, a point to be addressed in future studies.

Our investigations showed that RA SFB and OA SFB constitutively secreted considerable amounts of MMP-1 (20–40 ng/ml/30 h), with no significant differences between RA and OA. Incubation with TNF-α significantly stimulated MMP-1 secretion in both RA SFB (85-fold, roughly 2 µg/ml/30 h) and OA SFB (29-fold; 1 µg/ml/30 h; RA > OA), a process exclusively mediated by TNF-R55. In contrast to the results obtained for MMP-1, nonstimulated RA SFB and OA SFB secreted large amounts of TIMP-1, with no significant differences between RA and OA. Remarkably, however, RA SFB were completely unresponsive to TIMP-1 mRNA and protein induction by stimulation with TNF-α, while in OA SFB, TIMP-1 mRNA and protein were upregulated by TNF-α (2-fold, 5 µg/ml/30 h; OA > RA). The latter effect was mediated by stimulation of both TNF receptors. To our knowledge, there have been no reports on the specific role of individual TNF receptor types for TNF-α-induced TIMP-1 production in any fibroblast population. Moreover, this is the first study addressing the specific role of the individual TNF receptors for TNF-α-mediated production of the tissue-destructive MMP-1 in RA SFB.

The induction of MMP-1 mRNA and protein in RA SFB by stimulation with TNF-α is well known[42,43]. Induction of MMP-1 expression in human dermal fibroblasts by TNF-α is apparently also mediated exclusively by TNF-R55, i.e., only TNF-α and the agonistic anti-TNF-R55 Mab HTR-9 show an effect[44]. This emphasizes the pivotal role of TNF-R55 for the process of matrix remodeling/destruction in physiology and disease[45].

The strikingly divergent regulation of MMP-1 and TIMP-1 production in RA SFB by TNF-α, leading to an imbalance between destructive enzyme and its inhibitor in favor of the enzyme, has also been observed in conjunctival fibroblasts from patients with conjunctivochalasis or pterygium[46,47], in primary dentition periodontal ligament fibroblasts[48], endometrial fibroblasts[49], and, concerning the balance between MMP-9 and TIMP-1, also in human bronchial epithelial cells[50]. These findings indicate a strongly catabolic role of TNF-α in physiological and pathological conditions, while other cytokines, for example IL-6, appear to counterbalance this effect by inducing the production of inhibitors like TIMP-1 in synoviocytes and chondrocytes[51,52].

Of particular interest was the increase in TIMP-1 production in OA SFB by stimulation with TNF-α and with specific agonistic anti-TNF-R55 and TNF-R75 Mab, resulting in a much lower MMP-1/TIMP-1 ratio than in the case of RA SFB. Thus, the SFB of patients with RA may acquire alterations in the TNF-α signaling pathways that render them unresponsive to the induction of TIMP-1. Whether this involves alterations at the level of receptor expression or receptor affinity (in our study, expression of the TNF receptors did not differ significantly between RA and OA SFB), at proximal levels (e.g., altered ligand-receptor interaction as a result of oxygen radicals or cytokines) or at distal levels of intracellular NF-κB or AP-1 signal transduction[53-56], remains to be determined. Similarly, it remains to be determined whether this unresponsiveness applies to other stimuli inducing TIMP-1, including IL-6[51,52,57].

Our investigations show that RA and OA SFB constitutively secreted considerable amounts of IL-6. In both RA and OA SFB, stimulation with TNF-α significantly stimulated IL-6 secretion (OA SFB showing significantly higher values than RA SFB), an effect exclusively mediated by TNF-R55. Our findings concerning RA SFB are in good agreement with previous results, showing significant stimulation of IL-6 secretion by TNF-α, and exclusive stimulation of IL-6 secretion by agonistic Mab toward TNF-R55, but not by those toward TNF-R75[35]. These findings strongly argue in favor of predominant regulation of TNF-α-induced stimulation of IL-6 secretion by TNF-R55, in accord with the dependence of this stimulation on the NF-κB pathway[56] and the failure of TNF-α to induce the secretion of IL-6 via the NF-κB pathway in murine fibroblasts from TNF-R55-deficient mice[58]. The potential *in vivo* relevance of these findings is shown by reports describing the induction of IL-6 secretion in SFB co-cultured with TNF-α-producing U937 cells[59], and the reduction of IL-6 upon therapeutic TNF blockade for the treatment of severe RA[60].

Our experiments show that nonstimulated RA and OA SFB secreted only small amounts of PGE_2 (≤ 0.5 ng/ml), comparable to the levels previously reported for RA SFB, but much lower than the levels produced by RA synovial tissue explants *in vitro*[61]. TNF-α significantly stimulated the PGE_2 secretion in both RA SFB and OA SFB, a reaction mediated by both TNF receptors, however, with a predominance for TNF-R55. Stimulation through TNF-R75 was significantly more prominent in OA than in RA SFB. Similar to IL-6, our results clearly confirm studies in RA SFB[35,40] and OA SFB[62], in which TNF-α-mediated stimulation of PGE_2 secretion was found to be a function of ligand binding to both receptors, with predominance of TNF-R55, either by blocking assays using antagonistic Mab or by stim-

ulation with specific agonistic Mab toward individual TNF receptor types. Notably, TNF-α also induces the synthesis of cyclooxygenase 2 (COX-2), one of the enzymes involved in catalyzing the production of proinflammatory prostaglandins like PGE_2[63]. Since this effect is also predominantly mediated by TNF-R55 in OA SFB[62], these findings stress the pivotal proinflammatory role of the TNF-R55.

In this study, significant DNA fragmentation in RA SFB was induced exclusively by high concentration (25 µg/ml) of the agonistic anti-TNF-R55 Mab HTR-9, but not by stimulation with TNF-α (up to 50 ng/ml)[64]. However, the percentage of cells with DNA fragmentation after anti-TNF-R55 Mab exposure was very low (about 1.3%). Thus, the functional effects we observed, in particular the differential unresponsiveness of RA SFB to the TNF-α-induced increase of TIMP-1 production, were conceivably not influenced by ongoing apoptosis.

Our investigations show for the first time that significant DNA fragmentation above controls can be elicited in RA SFB by high concentrations of an agonistic Mab against TNF-R55. This finding is in good agreement with the known predominant role of TNF-R55 for induction of apoptosis in other cellular systems[65-67]. TNF-R55-mediated apoptosis may therefore contribute to the apoptosis observed in the RA synovial membrane *in vivo*[37,38], a mechanism possibly limiting or counteracting the proinflammatory effects of TNF-α in arthritis[68]. On the other hand, the failure of stimulation with TNF-α or with agonistic anti-TNF receptor Mab to induce significant DNA fragmentation in OA SFB indicates a differential sensitivity of RA SFB and OA SFB to the induction of apoptosis, as described in the case of Fas-mediated apoptosis[37,38].

Concurrent treatment of patients, e.g., with the use of potent corticosteroids, may have influenced the functional effects on SFB observed in our study[9]. Indeed, a positive correlation of steroid treatment was observed with the levels of MMP-1 protein induced by TNF-α (1 ng/ml; rho = 0.828, p = 0.042, n = 6), but a negative correlation was observed with the levels of TIMP-1 (10 ng/ml; rho = –0.828, p = 0.042, n = 6) and PGE_2 (1 ng/ml; rho = –0.840, p = 0.036, n = 6). Of interest as well, the rate of DNA fragmentation induced by high dose anti-TNF-R55 (25 µg/ml; rho = 0.956, p = 0.001, n = 7) correlated positively with steroid treatment. It remains to be clarified whether this indicates direct or indirect interactions of the treatment with TNF-α pathways and whether such effects may alter the efficacy of therapy.

It is somewhat difficult to explain why the individual stimulation of the 2 different TNF receptors by agonistic Mab *in vitro* results in different responses, although both receptors activate NF-κB and have at least partially overlapping signal transduction pathways[65]. Theoretically, separate stimulation of TNF receptors may lead to different intracellular complexes of IKK (IKK-ß, α, γ), which in turn result in differential activation of NF-κB-responsive genes involved in inflammatory responses and the induction/suppression of apoptosis[69]. However, it also has to be taken into consideration that one component of the physiological/pathophysiological action of TNF-α, the "ligand passing" from the TNF-R75 to TNF-R55, cannot be simulated with the specific anti-TNF receptor Mab used in the current investigations.

Considering all results, the TNF-R55 appears to be the major mediator of TNF-α-induced proinflammatory/tissue-destructive effector functions (e.g., MMP-1, IL-6, and PGE_2) in RA SFB, with little or no redundancy between the signal pathways of the 2 TNF receptors. Therefore, selective inhibition of TNF-α signaling through TNF-R55 in RA SFB, instead of broad neutralization of TNF-α by Mab or soluble TNF receptor constructs, may be a potential objective for antiinflammatory/antidestructive treatment of RA. The tools for selective inhibition of the TNF-R55 signaling pathway may become available in the near future, perhaps at the level of mutant ligands specific for a particular TNF receptor[70], by utilization of TNF receptor domains mediating ligand-independent receptor assembly[71], or by inhibitors of downstream signaling pathways with (partial) specificity for a particular TNF receptor[54,72]. Also, in contrast to the effects in RA SFB, distinct cellular functions in human articular chondrocytes were exclusively mediated by TNF-R75 (Alsalameh, *et al*, manuscript in preparation), possibly allowing targeting of different cell populations in the RA joint with some selectivity. Finally, selectively blocking the function of TNF-R55 (as in TNF-R55-deficient mice) may inhibit the noxious proinflammatory properties of TNF without compromising its immunosuppressive features[73,74].

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