Preferential Induction of Prodestructive Matrix Metalloproteinase-1 and Proinflammatory Interleukin 6 and Prostaglandin E₂ in Rheumatoid Arthritis Synovial Fibroblasts via Tumor Necrosis Factor Receptor-55

SAIFEDDIN ALSALAMEH, RAYYA J. AMIN, ELKE KUNISCH, HUGO E. JASIN, and RAIMUND W. KINNE

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 E₂ (PGE₂) 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 PGE₂ (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 PGE₂ (0.58 ng/ml; p ≤ 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 ≤ 0.05, OA > RA) and by separate stimulation of both TNF receptors. TNF-α-induced PGE₂ 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) by producing soluble proinflammatory mediators [e.g., interleukin 6 (IL-6) and prostaglandin E₂ (PGE₂)] and matrix-degrading enzymes [e.g., matrix metalloproteinase-1 (MMP-1)].

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Supported in part by grants from the Trinity Foundation and from the German Federal Ministry of Education and Research (FKZ 01ZZ9602).

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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. 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). 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).

TNF-α and its receptors play a major role in the pathogenesis of chronic inflammatory joint diseases such as human RA, as well as in several experimental arthritis models. 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. TNF-R75, while sharing some activities with TNF-R55 and enhancing signaling of the latter via “ligand passing,” 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.

In the RA synovial membrane, TNF-R55 and TNF-R75 are abundantly expressed in the lining layer, with a slight predominance of the latter. 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. 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.

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 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 PGE2 and the induction of DNA fragmentation in RA SFB and OA SFB were analyzed using specific agonistic Mab against both TNF receptor types.

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. 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, Egggenstein, Germany), as described. 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. SFB were > 89% prolyl-4-hydroxylyase+; > 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 × 105 SFB were suspended in 100 µl PBS/1% FCS/0.02% NaN3. 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 × 107 U/ml; endotoxin content < 0.1 ng/mg cytokine, LAL assay) was purchased from Genzyme (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. 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. Specificity of the Mab was confirmed by flow cytometry, Western blot, and immunoprecipitation as described. The HTR-9 Mab has agonistic effects in most TNF-R55 (CD120a) mediated signaling systems, whereas the UTR-1 Mab has agonistic properties in TNF-R75 (CD120b) mediated signaling. 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, and presumably reflecting blockade of “ligand passing.”

RA SFB or OA SFB (7th–9th passage, 2 × 105 cells/well; n = 3 each for determination of IL-6, PGE2, 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.

<table>
<thead>
<tr>
<th>Sex/Age</th>
<th>Disease Duration, yrs</th>
<th>RF</th>
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<th>CRP, mg/l</th>
<th>ARA Criteria for RA, n</th>
<th>Assay</th>
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<td>F</td>
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<td>10</td>
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<td>–</td>
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Table 2. Antibodies used in the study.

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<th>Main Cellular Expression/Ig Control</th>
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<td>IgG2a isotype control</td>
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<td>Soluble</td>
<td>Macrophages, fibroblasts, T cells</td>
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<td>Membrane</td>
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<td>TNF-R75</td>
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<td>Fas (CD95)</td>
<td>Membrane</td>
<td>Wide variety of cells</td>
<td>F/A</td>
<td>Immunotech</td>
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stimulated in duplicate cultures either with or without stimuli 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% CO2. 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 (BiotrakTM, 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 proform 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 × 105 cultured SFB using TRI Reagent™. The RNA was dissolved in water and treated with RQ1 RNase-free DNase (Promega, Madison, USA). Total RNA (1 µg) was reverse transcribed to cDNA using Superscript™ II reverse transcriptase (Gibco-BRL). 1 µg of cDNA was amplified in a final volume of 20 µl containing 1 × PCR buffer, 200 µM of each dNTP, 0.2 µM of each primer, and 1.25 units of Taq polymerase (Gibco-BRL). The reaction was performed in a DNA thermal cycler with the following programme: a 10-min denaturation step at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final extension at 72°C for 10 min.

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, Ludwigshaffen* |
| H98       | TNF-R55 (CD120a)   | Membrane              | Macrophages, fibroblasts, T cells  | F     | Dr. M. Gren, Inst. of Cell Biology and Immunology, Univ. of Stuttgart* |
| 80M2      | TNF-R75 (CD120b)   | Membrane              | Macrophages, fibroblasts, T cells  | A/T   | Dr. M. Brockhaus, F. Hoffmann La Roche Ltd., Basel, Switzerland |
| HTR-9     | TNF-R55            | Membrane              | Macrophages, fibroblasts, T cells  | A/T   | Dr. M. Brockhaus          |
| 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                |
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 α antibody with the color reaction.

Measurement of PGE₂. The concentration of PGE₂ was determined by comparison with a standard curve (range 39 to 5000 pg/ml). Due to the absence of a secondary anti-mouse antibody, interference of the TNF α antibody with 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 α antibody with the color reaction.

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 (0.6 ± 0.3%; Figures 1 and 2). 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 was detected on RA SFB or OA SFB (mean percentages < 0.4% in both cases; Figures 1 and 2). The Fas molecule was detected on RA SFB or OA SFB (mean percentages < 0.4% in both cases; Figures 1 and 2).

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. MPP-1: RA SFB and OA SFB (n = 3 each) constitutively secreted considerable amounts of MPP-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 MPP-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 MPP-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; 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 ≤ 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).

PGE₂: Nonstimulated RA SFB and OA SFB (n = 3 each)
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

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**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.
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 ≤ 0.05 for comparison between nonstimulated controls and TNF-α or TNF receptor-stimulated RA SFB; *p ≤ 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.
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. 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), confirming findings in *ex vivo* synovial tissue and mixed synovial cells from primary culture (data not shown). In the case of RA SFB, the ratio between TNF-R55 and TNF-R75 was higher than previously observed (roughly 12 vs 3–4). 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 ≤ 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 known42,43. 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 effect44. This emphasizes the pivotal role of TNF-R55 for the process of matrix remodeling/destruction in physiology and disease45.

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 pterygium46,47, in primary dentition periodontal ligament fibroblasts48, endometrial fibroblasts 50, and, concerning the balance between MMP-9 and TIMP-1, also in human bronchial epithelial cells50. 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 chondrocytes51,52.

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-kB or AP-1 signal transduction53-56, remains to be determined. Similarly, it remains to be determined whether this unresponsiveness applies to other stimuli inducing TIMP-1, including IL-651,52,57.

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-R7555. 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-kB pathway56 and the failure of TNF-α to induce the secretion of IL-6 via the NF-kB pathway in murine fibroblasts from TNF-R55-deficient mice58. 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 cells59, and the reduction of IL-6 upon therapeutic TNF blockade for the treatment of severe RA60.

Our experiments show that nonstimulated RA and OA SFB secreted only small amounts of PGE2 (≤ 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 vitro61. TNF-α significantly stimulated the PGE2 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 SFB5,40 and OA SFB62, in which TNF-α-mediated stimulation of PGE2 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, since this effect is also predominantly mediated by TNF-R55 in OA SFB, 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). 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. TNF-R55-mediated apoptosis may therefore contribute to the apoptosis observed in the RA synovial membrane in vivo, a mechanism possibly limiting or counteracting the proinflammatory effects of TNF-α in arthritis. 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 TNF-α-induced apoptosis, as described in the case of Fas-mediated apoptosis.

Concurrent treatment of patients, e.g., with the use of potent corticosteroids, may have influenced the functional effects on SFB observed in our study. 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 PGE₂ (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. Theoretically, separate stimulation of TNF receptors may lead to different intracellular complexes of IKK (IKK-β, α, γ), which in turn result in differential activation of NF-κB-responsive genes involved in inflammatory responses and the induction/suppression of apoptosis. 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 PGE₂) 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, by utilization of TNF receptor domains mediating ligand-independent receptor assembly, or by inhibitors of downstream signaling pathways (partial) specificity for a particular TNF receptor. 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.

ACKNOWLEDGMENT

Dr. A. Roth, Clinic of Orthopedics; Prof. Dr. G. Hein, Clinic of Internal Medicine IV; and Dr. W. Lungershausen, Department of Traumatology, Friedrich Schiller University Jena, are gratefully acknowledged for providing patient material and/or patient data. Bärbel Ukena for expert technical assistance; Dr. E. Birch-Hirschfeld, Institute of Virology, for primer synthesis. Prof. Dr. P. Scheurich, Dr. M. Grell, Institute of Cell Biology and Immunology, University of Stuttgart; and Dr. M. Brockhaus, Central Research Units, F. Hoffmann La Roche Ltd., Basel, Switzerland, are gratefully acknowledged for expert advice, revision of the manuscript, and for providing reagents; and Dr. Ernesta Palombo-Kinne for critical revision of the manuscript.

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