Interleukin-17, a regulator of angiogenic factor release by synovial fibroblasts

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Summary

Objective: Angiogenesis is a process stimulated in inflamed synovium of patients with osteoarthritis (OA), and contributes to the progression of the disease. Synovial fibroblasts secrete angiogenic factors, such as vascular endothelial growth factor (VEGF), an up-regulator of angiogenesis, and this ability is increased by interleukin (IL)-1β. The purpose of this study was to verify whether IL-17 contributes and/or synergizes with IL-1β and tumor necrosis factor (TNF)-α in vessel development in articular tissues by stimulating the secretion of proangiogenic factors by synovial fibroblasts.

Design: We stimulated in vitro synovial fibroblasts isolated from OA, rheumatoid arthritis (RA) and fractured patients (FP) with IL-17 and IL-1β and from OA patients with IL-17, IL-1β and TNF-α. In the supernatants from the cultures, we assayed the amount of VEGF by immunoassay and other angiogenic factors (keratinocyte growth factor, KGF; hepatocyte growth factor, HGF; heparin-binding endothelial growth factor, HB-EGF; angiopoietin-2, Ang-2; platelet-derived growth factor B, PDGF-BB; thrombopoietin, TPO) by chemiluminescence; semiquantitative RT-PCR was used to state mRNA expression of nonreleased angiogenic factors (Ang-2 and PDGF-BB) and tissue inhibitors of metalloproteinase (TIMP)-1.

Results: IL-17, TNF-α and IL-1β increased VEGF secretion by synovial fibroblasts from OA patients. IL-17 and IL-1β also increased VEGF secretion in RA and FP. Besides, IL-17 increased KGF and HGF secretions in OA, RA and FP; in OA and RA, IL-17 also increased the HB-EGF secretion and the expression of TIMP-1 as protein and mRNA. In OA patients IL-17 had an additive effect on TNF-α-stimulated VEGF secretion.

Conclusions: These results suggest that IL-17 is an in vitro stimulator of angiogenic factor release, both by its own action and by cooperating with TNF-α.

Key words: IL-17, Angiogenesis, Osteoarthritis, Rheumatoid arthritis, Inflammation.

Introduction

Osteoarthritis (OA) is a degenerative multifactorial disease with altered cartilage homeostasis, followed by synovial hypertrophy and hyperplasia, with proliferation of lining cells and subchondral bone alterations1. The synovium fibroblast-like cells (synovial fibroblasts) appear as cells activated secondarily by cartilage modification and do not show the invasive peculiarities and the tumor-like morphology found in rheumatoid arthritis (RA) patients2–8. However, in some cases, the morphological feature of synovitis found in OA can resemble that of RA, where synovial fibroblasts are activated cells, which show proliferating properties5. In OA and RA clinical symptoms, the synovial pannus feature, and the progression of the disease are increasingly associated with a synovium inflammation characterized by hypertrophy, proliferation of synovial cells, and also by the presence of infiltrate in sublining tissue9,10. Angiogenesis, i.e., the formation of new capillaries from preexisting vessels, is a process stimulated in joint tissues with chronic inflammation8, involving synovium and cartilage, the latter normally avascular, and its extent correlates with the progression of the disease6,9,10. A wide panel of factors up- or down-regulates the vessel proliferation and tissue invasion6. Many growth factors and cytokines can up-regulate the vascularization processes by directly inducing the migration and proliferation of endothelial cells to determine the expansion of small vessels11,12, and there are also indirect factors of angiogenesis, such as interleukin (IL)-6 and IL-1β, which elicit their effects by inducing tissue cells and/or attracting and inducing other cell types to the site of inflammation to secrete angiogenic factors13–16. Positive angiogenic factors are secreted by activated connective tissue cells6,17,18. Finally, other soluble factors mechanically facilitate or inhibit vascular growth and expansion by acting on the tissue integrity. Among the latter, tissue inhibitors of metalloproteinases (TIMPs) are modulated in articular tissues from RA and OA patients and can control angiogenesis by inhibiting the cartilage extracellular matrix degradation carried out by proteolytic enzymes, such as matrix metalloproteinases (MMPs)19,20.

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Vascular endothelial growth factor (VEGF) is one of the most important up-regulators of angiogenesis, and promotes endothelial cell migration and proliferation\(^\text{11}\). VEGF is a 46–49 kDa glycosylated homodimeric polypeptide, which has several variants. The main ones include 121, 145, 165, 189 and 206 amino acid isoforms. These isoforms present different abilities to bind to extracellular matrix proteoglycans and to VEGF receptors\(^\text{11}\). The expression of VEGF has been demonstrated in synovial fluid and membrane of RA and OA patients\(^\text{31,32}\) and is produced by synovial fibroblasts and activated macrophages\(^\text{17,23}\). We have recently demonstrated that proinflammatory cytokines such as IL-1\(\beta\), tumor necrosis factor (TNF-\(\alpha\)) and IL-17, over-expressed in articular RA and OA tissues\(^\text{1,24}\), also stimulate the secretion of VEGF by human synovial fibroblasts\(^\text{14,16}\), while the effect of TNF-\(\alpha\) is more debated\(^\text{4,16,26–28}\).

Thrombopoietin (TPO), keratinocyte growth factor (KGF), heparin-binding endothelial growth factor (HB-EGF), platelet-derived growth factor B (PDGF-BB), hepatocyte growth factor (HGF), and angiopoietin-2 (Ang-2) are also angiogenesis factors, which act directly and/or indirectly, by promoting the secretion of VEGF by dedifferentiated chondrocytes\(^\text{29}\). It is known that IL-1\(\beta\) is also able to stimulate the secretion of VEGF by synovial fibroblasts\(^\text{14,16}\). IL-17, over-expressed in articular RA and OA tissues\(^\text{1,24}\), is also able to stimulate the secretion of VEGF by synovial fibroblasts\(^\text{14,16}\)

The aim of our study was to verify the effect of IL-17 alone or in combination with IL-1\(\beta\) or TNF-\(\alpha\) on articular tissue angiogenesis, by stimulating synovial fibroblasts isolated from OA patients to secrete VEGF, and to see whether IL-17 can also stimulate the secretion of other positive or negative regulators of angiogenesis. The effect of IL-17 was also evaluated on synovial fibroblasts isolated from patients with RA and from patients who had undergone arthroplasty due to fracture.

**Materials and methods**

**Tissue samples and patients**

Synovial samples were obtained from patients with OA and RA undergoing knee or hip replacement surgery: 19 patients with OA (mean age 65 years, range 44–80 years) (eight knees and 11 hips). Diagnosis of OA was based on clinical, radiological and laboratory parameters\(^\text{32}\) and all OA patients involved in the study showed grade III–IV radiological features according to the Kellgren and Lawrence grading system\(^\text{33}\). For comparison, human synovium was obtained from four femoral heads and one tibial plateau resection, and the total cellular RNA was isolated with RNAwiz\(^\text{34}\) (Ambion, Austin, TX, USA) following the manufacturer’s instructions, and maintained at –80°C until its use.

**Isolation of Synovial Fibroblasts**

Specimens of synovial tissue were cut into small pieces (not more than 1 mm\(^3\)) and seeded in 25-cm\(^2\) culture flasks in Optimem-1 (Gibco BRL) supplemented with 200 μg/ml of gentamycin (Flow Laboratories, Biaggio, Switzerland), 15% heat inactivated fetal calf serum (FCS) (Gibco BRL) (complete medium) at 37°C in 5% CO\(_2\) humidified atmosphere, as previously described\(^\text{35}\). After about 15 days, when adherent cells from synovium pieces were at confluence, they were split by 0.05% trypsin/0.5 mM ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO, USA), washed and passed into a 75 cm\(^2\) flask (T 75). When synovial fibroblasts seeded in T 75 culture flask were confluent, they were tripinized and split 1:3 to obtain subsequent passages. Synovial fibroblasts were used between passages 3 and 8. At these passages synovial fibroblasts were phenotypically analyzed by a flow cytometer FACStar Plus (Becton Dickinson, St Diego, CA, USA) as previously described\(^\text{35}\) and appeared as a homogeneous population.

**Cell stimulation for angiogenesis factor release**

Synovial fibroblasts were cultured in a 96-well flat bottom microplate in 0.2 ml of complete medium (1 × 10\(^6\) cells/well). After overnight culture, 0.1 ml was substituted with 0.1 ml of complete medium with or without stimuli: 50 ng/ml recombinant human (rh) IL-17 (IL-17) (R&D Systems, MN, USA), 100 U/ml rhIL-1\(\beta\) (IL-1\(\beta\)); specific activity 5 × 10\(^7\) U/mg (Boehringer, Mannheim, Germany), and 100 U/ml rhTNF-\(\alpha\) (TNF-\(\alpha\); specific activity 1 × 10\(^8\) U/mg (Boehringer) (final concentration). Synovial fibroblasts were also stimulated with IL-17 added to different doses of IL-1\(\beta\) or TNF-\(\alpha\), as described for each experiment. After 72 h incubation, supernatants were collected and maintained at –80°C until their use. Stimulus concentration and incubation time were chosen on the basis of our previous experience with these cells and these cytokines\(^\text{25,35}\), mainly to obtain detectable amounts of not highly expressed soluble factors or optimal stimulation with TNF-\(\alpha\), which was a slower stimulus in comparison to IL-1\(\beta\) and IL-17 (data not shown). Fibroblasts from three OA patients were also cultured in complete medium for 10 h, then the complete medium was substituted with medium without FCS and, after 24 h incubation, stimuli, diluted in medium without serum, were added as above described.

**Cell stimulation for semi quantitative RT-PCR**

Synovial fibroblasts from five patients with OA were cultured in 6-well tissue culture plates in 4 ml of complete medium (2 × 10\(^5\) cells/well). After overnight culture, 2 ml was substituted with 2 ml of complete medium without or with stimuli: 50 ng/ml IL-17 (R&D Systems). After 5 h incubation, the supernatant was removed, the cells tri spunized, counted and the total cellular RNA was isolated with RNAwiz\(^\text{34}\) (Ambion, Austin, TX, USA) following the manufacturer’s instructions, and maintained at –80°C until its use.

**Enzyme-linked immunosorbent assay (ELISA) for VEGF and TPO**

VEGF and TPO secreted by nonstimulated and stimulated synovial fibroblasts were determined in the supernatant of 72 h culture by an ELISA with R&D Systems’ reagents. VEGF concentrations were evaluated using 0.4 μg/ml of capture antibody and 0.2 μg/ml of detecting antibody, following the manufacturer’s recommended protocol. These antibodies recognize VEGF\(_{121}\), VEGF\(_{165}\) and naturally-occurring human VEGF; so, most of the VEGF detected in our samples was VEGF\(_{121}\), the secreted isoform, soluble and not binding heparan sulfate or extracellular matrix\(^\text{11}\). The detection limit was 15 pg/ml. Streptavidin-HRP (Phar Mingen), diluted 1:1000, was used for the assay described,
and the substrate was tetramethylbenzidine (R&D Systems). Absorbance was measured at 450 nm, corrected by reading at 540 nm, by an ELISA reader (Labsystems, Helsinki, Finland). TPO concentrations were evaluated by a Quantikine™ kit for human TPO, following the manufacturer’s recommended protocol. The detection limit was 15 pg/ml.

CHEMILUMINESCENCE ARRAY FOR PROANGIOGENIC FACTORS AND TIMP-1

The Ang-2, HB-EGF, HGF, KGF, PDGF-BB and TIMP-1 concentrations were evaluated all together in the same sample by a SearchLight™ kit for Human Angiogenesis Array (Pierce Biotechnology, Rockford, IL, USA), following the manufacturer’s recommended protocol. The method is a sandwich ELISA test for multiple determinations, which utilizes a chemiluminescent substrate. The luminescent signal produced by enzyme–substrate reaction was detected with a SearchLight™ cooled CCD camera (Pierce Endogen, Woburn, MA 01801, USA). The detection limit was 20.6 pg/ml (Ang-2 and TIMP-1), 7.7 pg/ml (HB-EGF), 6.6 pg/ml (HGF) and 4.1 pg/ml (KGF and PDGF-BB).

SEMIQUANTITATIVE RT-PCR

One microgram of total RNA per sample was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (RT) (Perkin Elmer, Norwalk, CT, USA) and random examer priming, according to the manufacturer’s protocol (Perkin Elmer). One hundred nanograms of the various cDNAs was used in the subsequent amplification reactions. U937 cell line stimulated for 5 h with ionomycin (0.5 μM)/phorbol-myristate-acetate (PMA) (15 ng/ml)/lipopolysaccharide from E. coli (LPS) (0.5 μM) (Sigma) or PMA (10 ng/ml)/LPS (1 μg/ml) for Ang-2 and PDGF-B, respectively, and knee synovial fibroblasts from a subject with RA, for TIMP-1, were used as positive controls. Polymerase chain reaction (PCR) primers for Ang-2, PDGF-B and TIMP-1 were generated using the Light Cycler Probe Design Software (Roche Applied Science, Mannheim, Germany) from the Genbank sequences AF004327, NM_002608 and NM_003254, respectively, in order to span exons to discriminate amplification of genomic DNA, possibly contaminating RNA preparations: Ang-2 forward, 5'-CACCGATGGTGAACAGCG-3'; Ang-2 reverse, 5'-CTTGGACGAATAGCGTCT-3'; PDGF-B forward, 5'-GGTGAACATTCCAGGAC-3'; PDGF-B reverse, 5'-GGGCCATACGCAAATACCA-3'; TIMP-1 forward, 5'-CACCAAGACCTACGTG-3'; TIMP-1 reverse, 5'-GTGACGGGACTGGAAG-3'. Primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as endogenous control, were GAPDH forward, 5'-TGGTATCGTGGAAGGACTCATGAC-3'; GAPDH reverse, 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'. Real-time PCR for each transcript was performed using the QuantiTect SYBR Green PCR kit (QIAGEN GmbH, Hilden, Germany) in 20 μl final volume with 0.5 μM of each primer, 4.5 mM (Ang-2), 3.5 mM (PDGF-B) or 2.5 mM (TIMP-1 and GAPDH) MgCl2, in a Light Cycler instrument (Roche Molecular Biochemicals, Mannheim, Germany) with the following protocols: initial activation of HotStart Tag DNA polymerase at 95 °C for 15 min; 55 cycles (Ang-2 and PDGF-B) or 40 cycles (TIMP-1 and GAPDH) at: 94 °C for 15 s; 55 °C for 25 s (Ang-2) or 55 °C for 15 s (PDGF-B and TIMP-1) or 60 °C for 20 s (GAPDH); 72 °C for 15 s (Ang-2, TIMP-1 and GAPDH) or 72 °C for 25 s (PDGF-B). The crossing point values were determined for each sample and specificity of the amplicons was confirmed by melting curve analysis. To determine absolute GAPDH mRNA copy number, a standard curve was generated using 10-fold dilution series of gel-purified PCR products ranging from 6 × 10⁰ to 6 × 10⁵ copies. Amplification efficiencies for Ang-2, PDGF-B and TIMP-1, as calculated from the slopes of the log input amounts plotted vs crossing point values, were confirmed to be high and comparable with GAPDH amplification efficiency (differences varied between 2.2 and 3.2%). Relative mRNA expressions of Ang-2, PDGF-B and TIMP-1 were obtained by normalization to GAPDH by the formula \((1 + E)^{-C_{T1} - C_{T2}}\), where \(E\) represents the reaction efficiency (approximated to 1 because >90% for all the transcripts) and \(ΔC_T\), the difference between the GAPDH and the specific crossing point for each sample.

STATISTICAL ANALYSIS

Nonparametric tests were used for statistical analysis of the results: differences between groups were analyzed by the Mann—Whitney U test and when the Friedman analysis of variance (ANOVA) test for multiple comparison was significant, paired data were analyzed by the Wilcoxon matched-pairs test.

Results

EFFECT OF IL-17, IL-1β AND TNF-α ON THE SECRETION OF VEGF, TPO, KGF, HGF, HB-EGF AND TIMP-1 BY SYNOVIAL FIBROBLASTS

Fibroblasts isolated from synovium of OA, RA and FP patients maintained in culture with complete medium alone for 72 h (basal conditions) released VEGF, without differences among the groups (Fig. 1). IL-17 and IL-1β were able to

Fig. 1. Production of VEGF by synovial fibroblasts. Data are represented from synovial fibroblasts of five subjects with fractures (FP), seven RA subjects and 19 OA subjects. Results are expressed as pg/ml of VEGF secreted by synovial fibroblasts after 72 h culture under nonstimulated conditions (NS, white boxes), with IL-17 (50 ng/ml, hatched boxes), IL-1β (100 U/ml, light gray boxes) or TNF-α (100 U/ml, dark gray box, nine OA subjects). The symbols show the medians, the boxes show 25th and 75th percentiles, and the vertical lines below and above the boxes show 10th and 90th percentiles. Statistical comparison between nonstimulated and stimulated conditions of each group was made using the Wilcoxon matched-pairs test, and the Mann—Whitney U test was used to compare the different groups. *P < 0.05 and **P < 0.01 vs NS control of the same group.
increase the amount of VEGF secreted by synovial fibroblasts and showed comparable activity (Fig. 1). The stimulation of synovial fibroblasts from OA patients with TNF-α significantly increased the spontaneous release of VEGF compared to nonstimulated controls, such as the stimulation with IL-17 and IL-1β (Fig. 1). As we expected, in the absence of FCS, similar but reduced levels were obtained: the constitutive release of VEGF by synovial fibroblasts from three OA patients was (mean ± SD) 51 ± 28% lower than that in complete medium. The enhancing effects of IL-17, IL-1β and TNF-α, evaluated as relative increase, were lower than those obtained in complete medium, but significant (P < 0.05 vs nonstimulated control), with mean VEGF level increases of 1.8-fold, 2.2-fold and 1.3-fold, respectively, compared to 2.2-fold, 2.4-fold and 1.6-fold. This result may depend on the presence of some factors that induce VEGF production in FCS.

Spontaneous secretion of TPO by synovial fibroblasts was very low (range 20–54 pg/ml) and limited to nine of 23 (39%) OA, RA and FP patients, independently of the group studied. Similar results were obtained after stimulation with IL-17, IL-1β and TNF-α that could weakly modulate the TPO secretion in only some subjects from each group (OA, RA and FP) (data not shown).

Synovial fibroblasts from OA, RA and FP, secreted detectable amounts of KGF, HGF, and HB-EGF [Fig. 2(a–c)]. Interestingly, the amount of these angiogenic factors secreted by FP synovial fibroblasts tended to be higher than that secreted by RA and OA synovial fibroblasts, with significant differences for KGF and HB-EGF. The stimulation of synovial fibroblasts from RA and OA patients with IL-17 increased the spontaneous release of KGF, HGF and HB-EGF compared to nonstimulated controls. In FP patients, IL-17 increased the secretion of KGF and HGF, but not the release of HB-EGF. The effect of IL-17 seems to be mainly evident on synovial fibroblasts from subjects with important inflammatory components. In fact, when we compared the cytokine relative increase of each angiogenic factor after IL-17 stimulation between the different groups of subjects, we observed that IL-17 increased the release of KGF more on RA synovial fibroblasts than on FP and OA synovial fibroblasts (P = 0.009 and P = 0.003, respectively) [Fig. 2(a)]. Moreover, HGF release was increased more in RA than in OA synovial fibroblasts (P = 0.048) [Fig. 2(b)]; HB-EGF release was also increased more in OA than in FP synovial fibroblasts (P = 0.048) [Fig. 2(c)]. This analysis was performed by the Mann–Whitney U test.

Besides, synovial fibroblasts from all subjects secreted high amounts of TIMP-1, both under basal conditions, as expected, and after IL-17 stimulation. Nonstimulated samples with measurable, not very high, amounts of TIMP-1 are represented in Table I. The effect of IL-17 treatment tended to be stimulating but measurable only in one OA and three RA cases (Table I), in which the TIMP-1 level after IL-17 stimulation was not over-scale.

### IL-17 MODULATION OF mRNA SPECIFIC FOR ANG-2, PDGF-B AND TIMP-1

Since chemiluminescence array did not show detectable levels of Ang-2 and PDGF-BB in the supernatants, either from nonstimulated or IL-17-stimulated cultures, the possible IL-17 modulation of Ang-2 and PDGF-B mRNA expression was evaluated by real-time RT-PCR in synovial fibroblasts from four OA subjects. Synovial fibroblasts from three subjects incubated under basal conditions showed a very light signal for PDGF-B specific mRNA (relative expression range 0.1 × 10⁻⁵ to 2.04 × 10⁻⁵), which was more evident, but not significantly, after stimulation with IL-17. In one case, we did not observe the expression of PDGF-B specific mRNA, even in IL-17-stimulated
Secretion of TIMP-1 by synovial fibroblasts. Data are represented from nonstimulated samples with measurable amounts of TIMP-1: one of five subjects with fracture (FP), five of nine subjects with OA and four of five subjects with RA. Cells were incubated 72 h in medium alone (NS) or in the presence of IL-17 (50 ng/ml). All other nonstimulated and IL-17-stimulated samples secreted over-scale amounts of TIMP-1.

<table>
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<tr>
<th>Case</th>
<th>Disease</th>
<th>NS</th>
<th>IL-17</th>
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<tbody>
<tr>
<td>1</td>
<td>FP</td>
<td>15,920</td>
<td>&gt;33,000</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>2</td>
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<td>&gt;33,000</td>
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<tr>
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<td>OA</td>
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<td>&gt;167,000</td>
<td>&gt;1.2</td>
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<tr>
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<td>OA</td>
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<tr>
<td>5</td>
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<td>OA</td>
<td>137,748</td>
<td>&gt;167,000</td>
<td>&gt;1.2</td>
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<tr>
<td>8</td>
<td>RA</td>
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<td>&gt;167,000</td>
<td>&gt;1.2</td>
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<tr>
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*Stimulation index: ratio between TIMP-1 release by IL-17-stimulated cells and TIMP-1 release by cells cultured under basal conditions (NS).

**Over-scale sample.

cells. The maximum increment obtained was 2.4-fold, while nonstimulated U937 cells had PDGF-B mRNA relative expression of 0.98 × 10^-5 with an increment of 4.4-fold after stimulation. Similarly, Ang-2 specific mRNA was detectable in all subjects, although poorly expressed (relative expression range 1.76 × 10^-6 to 2.15 × 10^-5), but without significant modulation after IL-17 treatment. Nonstimulated U937 cells did not express Ang-2 mRNA, while after stimulation the relative expression was 16.84 × 10^-5. Also for TIMP-1, whose expression was very high in the supernatants, and in most cases the enhancing effect of IL-17 was not measurable, specific IL-17 mediated mRNA modulation was evaluated by real-time RT-PCR. TIMP-1 specific mRNA was highly expressed in all synovial fibroblasts tested and was up-regulated by IL-17 in three of four cases (Fig. 3). Case 1 (Fig. 3) also showed high amounts of TIMP-1 protein in the supernatant, positively modulated by IL-17 treatment (case 6, Table I).

**Fig. 3.** Relative expression of TIMP-1-specific mRNA by real-time RT-PCR in synovial fibroblasts from four subjects with OA. Relative TIMP-1 expression was obtained by normalization to GAPDH mRNA (see Materials and methods) in synovial fibroblasts after 5 h culture under nonstimulated conditions (NS, light gray boxes) and stimulated with IL-17 (50 ng/ml, dark gray boxes). Case 1 corresponds to case 6 in Table I.

**Fig. 4.** Effect of IL-17 on the secretion of VEGF by synovial fibroblasts costimulated with IL-1β or TNF-α. Results are represented as the median (symbols) of pg/ml of VEGF secreted by synovial fibroblasts obtained from five subjects with OA and incubated 72 h under basal conditions (NS), with IL-17 (50 ng/ml), IL-1β (100 U/ml), TNF-α (100 U/ml) or IL-17 combined with IL-1β or TNF-α at various concentrations, as indicated in the graph (1-10-100 U/ml). The boxes show 25th and 75th percentiles, and the vertical lines below and above the boxes show 10th and 90th percentiles. Statistical comparison between nonstimulated and stimulated conditions of each group was made using the Wilcoxon matched-pairs test, and the Mann–Whitney U test was used to compare the different groups.

*P < 0.05 and **P < 0.01 vs NS controls; †P < 0.05 vs IL-17, IL-1β and TNF-α alone.

Discussion

IL-17 has been recently described as a proinflammatory cytokine secreted by T-activated lymphocytes (reviewed by Gaffen37). This cytokine is over-expressed in OA and RA joint tissues, and, although the precise site of IL-17 production is still unclear, it may be considered as a factor that contributes, with IL-1β and TNF-α, to the pathogenesis of rheumatic diseases1. Recently, we have reported that synovial fibroblasts and chondrocytes from OA patients express the receptor of IL-1735, and we have described the stimulating effect and, moreover, the amount of VEGF was lower than the sum of the amounts stimulated by IL-17 and TNF-α alone (Fig. 4).

EFFECT OF IL-17 ON VEGF SECRETION STIMULATED BY IL-1β OR TNF-α

We also investigated the combined effect of IL-17 with IL-1β or TNF-α on VEGF secretion. Synovial fibroblasts from five OA patients were utilized to control the additional effect of IL-17 on cultures stimulated with various concentrations of IL-1β or TNF-α, as specified (Fig. 4).

IL-17 did not affect the release of VEGF stimulated by IL-1β, at any of the concentrations tested, but the additional presence of IL-17 in cultures stimulated with TNF-α was able to induce a higher secretion of VEGF, in comparison with the stimulation with TNF-α alone. This increment was significantly higher only at the highest concentration of TNF-α. IL-17 and TNF-α had an additive, nonsynergistic effect and, moreover, the amount of VEGF was lower than the sum of the amounts stimulated by IL-17 and TNF-α alone (Fig. 4).
tissues exhibited VEGF positive cells, which were very rare in normal synovial tissues. Normal human synovial fibroblasts secrete in vitro lower levels of VEGF than RA or OA synovial fibroblasts. This evidence supports the role of VEGF in promoting angiogenesis in the synovium. We found that not only IL-1β, IL-17, and TNF-α increase VEGF secretion by synovial fibroblasts from OA, RA and FP patients. Previously it had been shown that the effect of TNF-α might be undetectable up to 24–48 h of incubation, or depending on the culture conditions, such as cell density, and mainly, the cell source.

The effect of the TNF-α-stimulated VEGF secretion was enhanced by IL-17, which had no effect on IL-1β stimulated cells. The amount of VEGF secreted after stimulation with IL-17 plus TNF-α never exceeded the sum of the amounts stimulated by IL-17 and TNF-α separately. Therefore, it seems that in our experimental model IL-17 exerted an additive but not synergistic effect on TNF-α. It may be hypothesized that the link of IL-17 and TNF-α to their own receptor on synovial fibroblasts activates the synthesis of VEGF by independent metabolic pathways, because of the additive effect of these two cytokines, whereas IL-17 and IL-1β probably utilize activation pathways that interfere with each other. Recent studies have demonstrated that VEGF expression is regulated by the activation of transcription nuclear factor-kB (NF-kB). IL-17, IL-1β and TNF-α, as many inflammatory cytokines, can modulate the gene expression by NF-kB, although it is known that IL-17 can also utilize other signaling pathways. So, the cytokine-stimulating effect might depend on the combination of the signal transduction and transcription pathways utilized by the cytokine target cells.

Interestingly, synovial fibroblasts from subjects with fractures (FP) constitutively secreted VEGF in vitro, and in amounts comparable to those found in OA and RA, although these subjects did not present any articular morphological alterations. It is likely that VEGF secretion in FP patients is a self-limited phenomenon related to the posttrauma inflammation, in accordance with the presence of some biochemical parameters of inflammation, such as elevated level of protein C reactive and erythrocyte sedimentation rate (VES) in the patients included in this study, and our observations of the presence of IL-1β and TNF-α positive chondrocytes in posttraumatic patients.

Although VEGF seems to be one of the most important inducers of vessel proliferation, and is also one of the most widely studied, there are many different growth factors involved in angiogenesis. We previously showed that IL-17 can increase IL-8 release by synovial fibroblasts from OA patients. IL-8 is a chemokine able to stimulate the proliferation of endothelial cells. In accordance with these findings, IL-17 can up-regulate the constitutive release of other important angiogenesis factors, such as KGF and HGF. HB-EGF is a direct/indirect angiogenic factor, which can stimulate the endothelial cell proliferation more potently than other angiogenic factors, such as basic fibroblast growth factor (b-FGF), VEGF and IL-6; moreover, its effect is additive with b-FGF. HB-EGF is a direct/indirect angiogenic factor, being able to stimulate the proliferation of microvascular endothelial cells and vessel smooth muscle cells and the secretion of VEGF and metalloproteinases. It is not surprising that synovial fibroblasts from FP subjects released higher amounts of HGF than RA and OA synovial fibroblasts at basal culture conditions, because HGF is present in normal synovial tissues, and the percentages of HGF-positive fibroblasts seem to be higher in normal samples than in RA and OA synovial specimens. In this study cultured synovial fibroblasts appeared to maintain the same phenotype as in vivo, releasing more HGF when isolated from nonarthritic than from arthritic tissues. Because it is not known whether there are KGF- and HB-EGF-positive cells in synovial membrane, or whether synovial fibroblasts can release these factors, KGF and HB-EGF could be released by cultured synovial fibroblasts in a similar way to HGF. So, IL-17 appears as an in vitro potent proangiogenic cytokine.

In addition, we have shown that synovial fibroblasts, cultured in the presence of IL-17, increased their constitutive TIMP-1 expression. TIMP-1 can be defined as an inhibitor of angiogenesis, having MMPs inhibitory activity. It has also been shown that TIMP-1 inhibits migration and tubular formation in vitro by microvascular endothelial cells. Our findings are not surprising, because TNF-α is also able to stimulate both VEGF and TIMP-1 by synovial fibroblasts. The possible increasing effect of IL-17 on TIMP-1 expression by OA synovial fibroblasts could represent a model of autoregulation: IL-17 could dampen its own excessive proangiogenic activity by also stimulating the secretion of soluble factors able to oppose the angiogenesis.

Although IL-17 can up-regulate several main angiogenic factors, in our experimental model this cytokine seems unable to stimulate the expression of TPO, PDGF-BB and Ang-2, and appears as a proangiogenic cytokine able to stimulate a limited panel of angiogenic factors, thus improving more than inducing their gene expression.

In conclusion, our findings support the hypothesis that IL-17 is an important factor correlative for the pathogenesis of OA and RA, but further studies are necessary to understand the fine mechanisms activated by this cytokine to identify new therapeutic targets to control inflammation and angiogenesis.

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**References**


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