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SHORT COMMUNICATION

Angiogenic growth factors in rheumatoid arthritis

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Abstract We investigated whether the angiogenic profile, which is based on the local expression and systemic levels of angiogenic growth factors (VEGF, Ang-1, Ang-2, and the corresponding receptors), differs between rheumatoid arthritis (RA) and osteoarthritis (OA) patients. We determined the expression of VEGF, Ang-1, and Ang-2 together with its receptors (VEGFR-1/-2 and Tie2) in synovium tissue (ST) and muscular tissue (MT) from patients with RA and OA using quantitative PCR. Tissue samples were obtained from 15 RA and 19 OA patients during total knee arthroplasty. Control MT samples ($n = 10$) were obtained during spinal surgery. Results are correlated to VEGF and angiopoietin serum levels via ELISA measurements. The VEGF expressions in ST and serum levels were significantly higher in RA patients than in OA patients ($P < 0.05$). Furthermore, the VEGFR-1 and VEGFR-2 expression in ST from RA patients were significantly higher than in OA patients ($P < 0.001$ and $P < 0.05$). The relative concentration of angiopoietins (Ang-1/Ang-2 ratio) was significantly increased in RA ($P < 0.01$). Serum levels for Ang-2 showed no significant differences. Statistical analysis showed a significant higher level of Tie2 in RA patients ($P < 0.001$). Analysis of local levels of VEGF, VEGFR-1, VEGFR-2, Ang-1, Ang-2, and Tie2 in the muscular tissue showed no significant difference between RA and OA patients. These results underline the importance of pro-angiogenic growth factor levels for RA corroborating the assumption that VEGF and angiopoietins play an important role in the pathogenesis of RA.

Keywords Angiogenesis · Rheumatoid arthritis · Vascular endothelial growth factor · Angiopoietin

Abbreviations

Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
Tie2	TEK receptor tyrosine kinase
SEM	Standard error of mean
VEGF	Vascular endothelial growth factor
PDGF	Platelet-derived growth factor
FGF	Fibroblast growth factor
TGF-alpha	Transforming growth factor-α

Introduction

Angiogenesis is not only necessary for maintaining physiologic functions, such as wound healing and embryonic

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development, but also plays a pivotal role in different pathologies, like tumorigenesis, diabetic retinopathy, and the formation of pannus tissue in rheumatoid arthritis [1, 2].

RA is characterized by chronic inflammation of the joints. Infiltration by plasma cells, lymphocytes, and macrophages together with hyperplasia of synovial cells results in the overgrowth of a fibrovascular granulation tissue, known as pannus, and ultimately results in the progressive destruction of cartilage and bone [3]. Besides the well-established importance of pro-inflammatory cytokines like IL-1 and TNFα, recently the role of angiogenesis for the continual proliferation of synovial tissue and therefore, the progression of RA, has been stated [4]. Various angiogenic factors have been related to RA, but little is known about the site-specific expression of angiogenic growth factors in different tissues involved in rheumatoid arthritis.

Newly formed blood vessels require the recruitment of surrounding mesenchymal cells and their differentiation into vascular smooth muscle cells to become stable vascular structures. Vessel maturation is facilitated by the recently identified angiopoietins. Ang-1 accounts for the recruitment of surrounding mesenchymal cells and promotes their differentiation into vascular smooth muscle cells, which significantly enhances vascular stability of new blood vessels. In contrast, Ang-2 has been identified as a natural antagonist of Ang-1 and can inhibit angiogenesis. Thus, the formation of stable neovascular vessels requires endothelial cell migration and proliferation, mediated by vascular endothelial growth factor (VEGF), but also the coordinated actions of the different angiopoietin molecules for the stabilization of the vessel wall [5].

Due to the critical importance of Ang-1 for the later stages of neo-angiogenesis and maturation of blood vessels, we hypothesized that the expression of Ang-1 in synovial tissue is required for angiogenesis in rheumatoid arthritis. To further elucidate the influence of VEGF and angiopoietins on the local and systemic level, we performed quantitative measurements of these growth factors and receptors in RA and OA patients.

Objective of the present study was to determine whether in both RA and OA, a stimulated angiogenic path of ST and MT is involved. A comprehensive characterization of the angiogenic balance should help to distinguish between a local activation via local inflammation versus systemic activation. Local activation, like in OA, could require a local therapy while stimulation on different sites could require a systemic therapy.

Materials and methods

Patients were recruited from the Department of Orthopedic Surgery, Klinikum Bad Bramstedt, Bad Bramstedt, Germany. According to the American College of Rheumatology criteria, patients were diagnosed with RA or OA [6]. Tissue samples were obtained during total knee arthroplasty from both collectives. Written consent was obtained from patients, and all procedures were performed under surveillance of the local ethics committee.

Tissue samples were collected from 15 patients with RA (3 men and 12 women; mean age 55 years, range, 31–76 years, mean duration of disease of 15.25 ± 4.44 years), 19 patients with OA (14 men and 5 women; mean age 61 years, range, 55–71 years). Synovium tissue samples were collected in the course of a total knee arthroplasty operation and kept frozen below $-80°C$ until measured. Furthermore, from a larger collection (VEGF: RA $n = 41$, OA $n = 54$; Ang-2 RA $n = 30$, OA $n = 35$) of patients presenting similar characteristics, serum samples were obtained.

Quantitative PCR

For quantitative PCR, total RNA was reverse-transcribed using random hexameric primers and Superscript reverse transcriptase (Invitrogen, Germany). cDNAs were quantified relatively by real-time PCR on a LightCycler using specific primers for human VEGF, VEGF-Receptor 1, VEGF-Receptor 2, Angiopoietin-1, Angiopoietin-2, human Tie 2, and s27. The thermal cycler parameters and primer sequences are available on request. Quantification was done using the second derivative maximum method of the LightCycler software (Roche, Germany). Levels of VEGF, VEGFR-1, VEGFR-2, Angiopoietin-1, Angiopoietin-2, and Tie2 were subsequently normalized to the housekeeping gene s27, and relative quantification with efficiency correction was performed with the LightCycler Software (Roche, Germany). All measurements were performed at least twice. Results are means of mRNA levels of the different measurements.

VEGF serum ELISA

Serum levels of VEGF and Angiopoetin-2 were measured by enzyme-linked immuno-sorbent assay using a commercially available (ELISA) kit (R&D Systems, VEGF DVE00, Ang2 Cat.-No DANG20).

Statistical analysis

Statistical analysis was performed with SPSS Software (SPSS, Chicago, IL). Comparisons between the different patient groups were made using the Mann–Whitney *U* test.

A value for $P < 0.05$ was considered statistically significant. PCR results are presented as relative expression compared to RA.

Results

Relative synovial tissue VEGF expression was significantly lower in OA patients (OA = 0.67 ± 0.12, $P < 0.01$, Fig. 1a). The mean tissue VEGFR-1/FLT-1 level was significantly lower in OA than in RA patients (OA = 0.43 ± 0.044, $P < 0.01$, Fig. 1b). Quantitative measurements of VEGFR-2/FLK-1 resulted in significant lower mean levels in OA patients than in RA patients (OA = 0.40 ± 0.048, $P < 0.05$, Fig. 2a).

The simultaneously collected muscular tissue samples were analyzed for VEGF, VEGFR-1, VEGFR-2, Angiopoietin-1, Angiopoietin-2, and Tie2. No significant differences were found between RA and OA patients.

Significantly lower levels of the angiopoietin receptor Tie2 were found in ST samples from OA patients (OA = 0.43 ± 0.044, $P < 0.01$) (Fig. 2b).

Serum concentration of VEGF was found to be significantly higher in samples from RA patients than in OA patient probes (RA = 457.9 ± 58.3 pg/ml, OA = 300.34 ± 35.6 pg/ml, $P < 0.05$) (Fig. 3a).

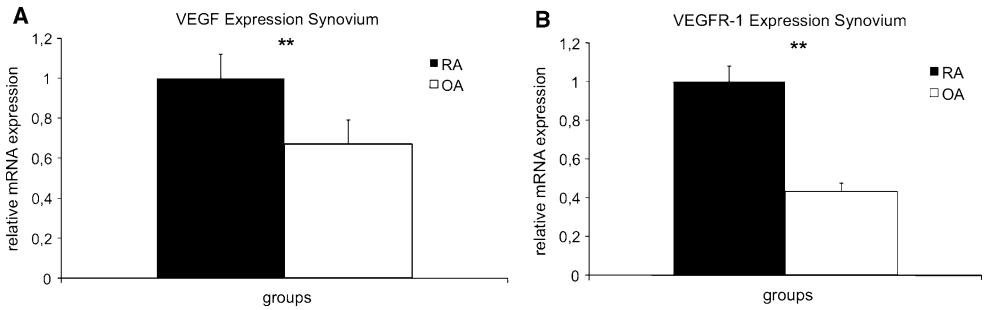

Fig. 1 **a** Relative expression of VEGF in the synovial tissue samples. Statistical significant increase in RA patients ($P < 0.01$). **b** Relative expression of VEGFR-1 in the synovial tissue samples. When compared to OA patients, a statistical significant increase in RA patients ($P < 0.001$) was found. *Bars* represent expression ±SEM

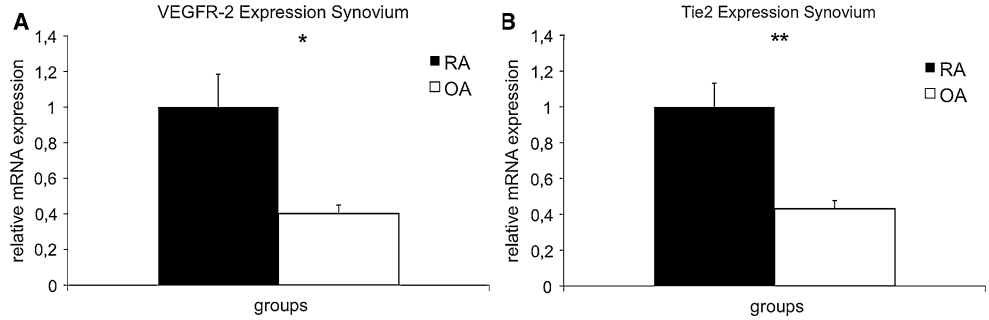

Fig. 2 **a** Relative expression of VEGFR-2 in the synovial tissue samples. We found a statistical significant increase in RA patients ($P < 0.05$). **b** Relative expression of Tie2 in the synovial tissue samples. Statistical significant increase was found in RA patients ($P < 0.001$). *Bars* represent expression ±SEM

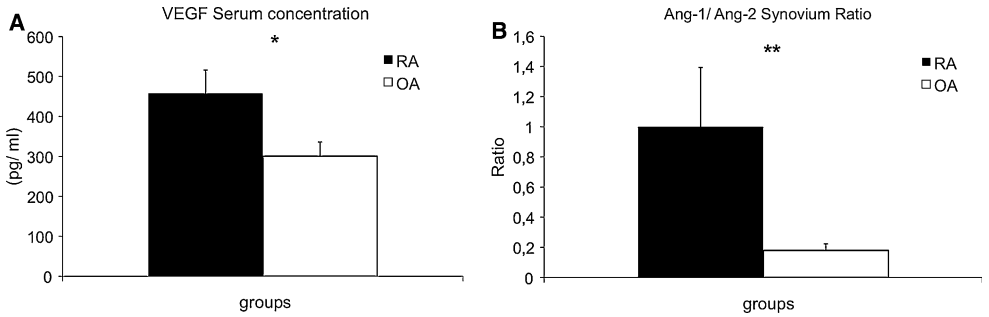

Fig. 3 **a** Serum levels of VEGF. A statistical significant increase in RA patients ($P < 0.05$) was found when compared to OA patients. *Bars* represent serum levels ±SEM. **b** Ratio between synovial tissue levels of Ang-1 and Ang-2. Statistical significant increase in RA patients ($P < 0.05$) is observed. *Bars* represent mean synovial tissue level ratio ±SEM

Since Ang-1 and Ang-2 are competing for binding to Tie2, we calculated Ang-1/Ang-2 ratios to determine balance of the angiopoietin–Tie system. While in the muscular samples, no significant differences for Ang-1/Ang-2 ratio were found, significant higher ratios of Ang-1/Ang-2 were found in ST of RA patients ($P < 0.01$) (Fig. 3b). Serum levels for Ang-2 showed no significant differences between both groups (RA = 1,970 ± 167 pg/ml; OA = 2,072 ± 176 pg/ml).

Discussion

VEGF, also known as vascular permeability factor, is a secreted protein that acts on endothelial cells via interaction with its receptors and is described as the most potent pro-angiogenic growth factor [7]. VEGF acts as a growth stimulus and as a survival factor for endothelial cells. The important role of VEGF for an increased vascular permeability results from capillary fenestrations, decreasing of intercellular junctions and building of transcellular gaps [8]. This leads to barriers to drug delivery and malignant effusions in tumor patients and joint swelling in RA patients [9]. Different authors described increased levels of serum and synovial fluid VEGF in RA patients when compared with OA or other forms of arthritis, indicating that the main producers of this cytokine were macrophages and synovial lining cells [10, 11]. In our study, the synovial tissue and serum VEGF concentrations were significantly higher in RA patients than in OA patients (Figs. 1a, 3a). Furthermore, the levels of both measured VEGF-Receptors VEGFR-1 and VEGFR-2 were significantly increased in RA patients (Figs. 1b, 2a). These results corroborate former studies [12–14]. Expression of VEGF and its receptors in the muscular did not show significant differences between RA and OA patients.

There is increasing evidence that suggests a complex interaction between VEGF and the angiopoietin/Tie2 family and its involvement in rheumatoid arthritis [15, 16]. Angiopoietins are critical for neovascularization and function of newly formed vessels. Ang-1/Tie2 binding induces stabilization and maintenance of maturing blood vessels. In contrast, Ang-2 antagonizes Ang-1, stimulating vascular invasion and blocking maturation/stabilization in the presence of abundant VEGF. These vessels remain in a moldable condition where, in response to VEGF, they increase in capillary diameter, remodel, and new blood vessels sprout [17]. We found drastically increased ratios of Ang-1/Ang-2 in synovial tissue samples from RA patients while no differences were found in the muscular tissue samples. This depicts the accelerated vascular overturn and pro-angiogenic status, which characterizes rheumatoid arthritis. While confirming the results of former studies regarding the angiopoietin/Tie2 system in synovial tissue, no significant alterations were found in the muscular tissue. [18, 19] Recently, the usage of tyrosine kinases inhibitors for treatment of RA has been suggested as a possible therapeutic alternative [20]. The presented data showing an increased expression of the endothelium-specific receptor tyrosine kinase Tie2 and the corresponding agonist Ang-1 in synovium tissue samples from RA patients seem to further underline this assumption. We observed a trend toward elevated levels of serum Ang-2 in OA patients without significant differences between both groups ($P = 0.056$). The role of angiopoietins for development of osteoarthritis still remains unclear, and further investigation is required to better understand the complex interaction between inflammation, matrix turnover, and angiogenesis, which leads to the structural damage in osteoarthritis [21]. Furthermore, from the site-specific differences in the expression of angiopoietins, possible consequences for therapeutic approaches might result, which should be further investigated.

Conclusion

A comprehensive characterization of angiogenic growth factors in rheumatoid arthritis is presented corroborating former studies. The angiopoietins and the corresponding tyrosine kinase receptor Tie2 are constitutively expressed in the synovium of RA patients. No elevation of angiogenic growth factor expression was found in the musculature. Inhibition of Tie2 activation and modulation of angiopoietin expression/function seem to be favorable targets for the treatment of RA in the synovium tissue.

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