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Original article

Linking systemic angiogenic factors (VEGF, angiogenin, TIMP-2) and Doppler ultrasound to anti-inflammatory treatment in rheumatoid arthritis

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ABSTRACT

Objective: To evaluate an association between synovial Doppler flow and serum levels of vascular endothelial growth factor (VEGF), angiogenin and TIMP-2 in patients with rheumatoid arthritis during anti-inflammatory treatment with glucocorticoids and TNF-α inhibitors.
Methods: Inflamed wrists of 15 patients with rheumatoid arthritis (RA) were examined by two independent ultrasound investigators prior to and at days 3, 7, 14 and 42 after the initiation of treatment with glucocorticoids in therapy-naïve patients or after the beginning of a therapy with a TNF-α inhibitor in patients with DMARD failure. Quantitative three-dimensional power Doppler ultrasonographic assessment of synovial vascularization was compared at each visit with serum levels of VEGF, angiogenin and TIMP-2.
Results: In the glucocorticoid group, synovial Doppler signals decreased significantly at day 3 (−44%; $P = 0.003$) in comparison to a delayed decrease in the TNF-α inhibitor group after 6 weeks (−46%; $P = 0.001$). A significant reduction of serum VEGF levels could be determined with a delay of 1 week after the decrease of Doppler activity but no correlation was found between both parameters (rho: $P = 0.7$; $r = -0.03$). Angiogenin concentrations decreased in the TNF group and increased in the GC group. Levels of TIMP-2 did not change significantly in both groups.
Conclusion: The decrease of serum VEGF levels under treatment with glucocorticoids or TNF-α inhibitors followed the reduction of the intra-articular synovial Doppler flow. This result supports the idea that the reduction of synovial perfusion due to anti-inflammatory treatment is not regulated by systemic VEGF, but that the inflamed joints are the source for circulating VEGF.

1. Introduction

In rheumatoid arthritis (RA), bone and cartilage destruction is caused by an extensive growth of synovial pannus, which is dependent on an accompanying neovascularisation to nourish and oxygenate the increased tissue mass. Formation of new microvessels from the preexisting vasculature consists of multiple processes such as degradation of vascular basement membranes and surrounding extracellular matrix as well as migration and proliferation of endothelial cells. This complex is driven by a combination of up-regulation of angiogenic promoters, and down-regulation of inhibitors [1,2].

The vascular endothelial growth factor (VEGF) is one of the most important and best-known agents to stimulate angiogenesis in different disorders such as cancer or arthritis [3–6]. In the synovial tissue of patients with RA, the localization of VEGF polypeptide could be demonstrated in macrophages and fibroblasts surrounding microvessels, vascular smooth muscle cells and synovial lining cells [7]. In patients with RA, higher serum VEGF concentrations are reported compared to healthy controls. The elevated serum VEGF levels can be derived from different sources such as the inflamed synovial tissue, synovial fluid neutrophils, peripheral blood mononuclear cells or platelets [8,9].

Angiogenin is a member of the ribonuclease superfamily, which was initially isolated from cultured tumor cells. It is another potent inducer of the neovascularization and appears to be responsible for the stabilization of the blood vessels during the formation of a new microvasculature in different conditions. Elevated levels of angiogenin concentrations were found in synovial fluid of patients with rheumatoid arthritis, and it could be shown that angiogenin protein was released by cultured synovial fibroblasts [10,11].

Matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP) are enzymes with a broad range of different

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functions which are involved in the process of angiogenesis with respect to matrix remodeling. The measurement of serum and synovial MMP and TIMP of patients with inflammatory and degenerative joint disorders confirmed their role as indicators for the extent of tissue damage as well as for the inflammatory activity [12,13].

Angiogenesis describes the process of new blood vessel formation, which leads, in combination with the inflammation-induced vasodilation of preexisting blood vessels, to an increase of intra-articular perfusion in the affected joints. Here, numerous studies in recent years demonstrated the substantial potential of highly sensitive power Doppler Ultrasound (PDUS) to improve the evaluation of treatment response in RA patients by visualizing the expanded synovial vasculature as result of the increased angiogenesis during pannus formation [14–17]. However, the mode of action, how different treatments such as GC, conventional disease modifying anti-rheumatic drugs (DMARD), or biologics are able to reduce the synovial perfusion, is not well understood. Some studies demonstrated a significant decrease of serum VEGF levels during anti-inflammatory treatment with conventional DMARD or biologics such as TNF-α inhibitors or anti IL-6 therapy [18–21]. Other cross-sectional studies that correlated serum vascular markers with intra-articular power Doppler activity provided contradictory results [22–24]. No data is currently available for the association of systemic angiogenic activity, measured by angiogenic factors in the serum and the amount of local synovial vascularization, measured by Doppler ultrasound, in sequential studies under different treatment strategies.

Therefore, the aim of this study was to evaluate the kinetics of serum vascular markers (VEGF, angiogenin, TIMP-2) and synovial vascularity (PDUS) during anti-inflammatory treatment in order to elucidate the role of systemic angiogenic factors inside the regulation of angiogenesis in arthritis.

2. Methods

Fifteen RA patients, who fulfilled the American College of Rheumatology 1987 revised criteria and presented with an active arthritis in at least one wrist, were enrolled in this study. The respective demographic and clinical data has already been published in a technical evaluation of three-dimensional PDUS examination [25]. Nine out of these 15 patients had no prior immunosuppressive anti-rheumatic therapy, and a first-line treatment with GC was started (GC group). In five out of these nine patients, a DMARD therapy with methotrexate (MTX) was additionally initiated. The other six patients had a longstanding RA for more than 10 years and required initiation of a new therapy with a TNF-α inhibitor (infliximab, etancercept or adalimumab). Treatment decision and DMARD selection for the individual patient was independent from this study.

Clinical disease activity parameters such as DAS 28 and HAQ and laboratory markers such as CRP and ESR were assessed before the new treatment with GC or TNF-α inhibitor was started (visit 1), thereafter at day 3 (visit 2), day 7 (visit 3), day 14 (visit 4) and at day 42 (visit 5), resulting in 5 sequential visits in every patient. Besides, a complete ultrasound investigation of the dominant wrist including grey scale and PDUS was performed. For the quantification of Doppler activity, a new method as recently demonstrated, was used [25]. In short, three-dimensional (3D) image volumes of the synovial vascularity were quantified by an image analysis program [26], resulting in a number of "voxels". Voxel is short for "volume pixel", representing the smallest distinguishable box-shaped part of a three-dimensional image. Thus, a very exact 3D measurement was available for the synovial microvascular Doppler flow and could be used for further analyses.

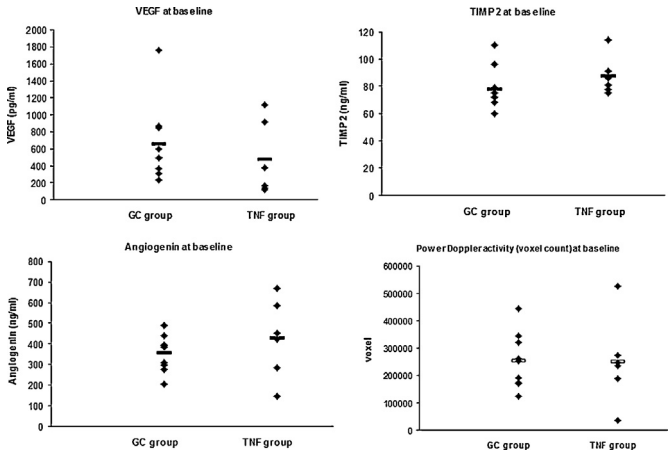

Fig. 1. Baseline values of VEGF, angiogenin, TIMP 2 and Doppler activity (voxels) in the glucocorticoid and in the TNF group. ANG: angiogenin; GC: glucocorticoid; TNF: anti-TNF alpha therapy.

The blood samples for the measurement of the angiogenic factors VEGF, angiogenin and TIMP-2 were centrifuged and the serum was stored at −80 °C until further analysis. VEGF, angiogenin and TIPM-2 levels were measured using a standard quantitative sandwich ELISA according to the manufacturers' instructions for serum measurements (R&D Systems, Wiesbaden-Nordenstadt, Germany). At the end of the test, optical densities were determined with a microplate reader (TECAN Deutschland GmbH, Crailsheim, Germany). VEGF concentrations are reported as mean values in pg/mL, angiogenin and TIMP-2 levels in ng/mL.

The protocol was approved by the local ethics committee of the Justus-Liebig university of Giessen. A written informed consent was obtained from each patient according to the Declaration of Helsinki.

The comparison of findings at baseline and follow-up were evaluated using Wilcoxon's rank sum test. For the correlation between serum markers, DAS 28 and PDUS voxel count, Spearman's rank test was used. The level of significance was regarded as $P < 0.05$.

3. Results

Baseline VEGF levels were higher in patients with no previous treatment (661 pg/mL versus 471 pg/mL), whereas angiogenin concentrations were slightly higher in patients with longstanding RA. TIMP-2 levels and pretreatment Doppler activity (voxel count) were almost equal in both treatment groups (Fig. 1).

The Doppler activity decreased significantly until day 3 (−44% from pretreatment values; $P = 0.003$) in the GC group in comparison to a more delayed decrease in the TNF group at day 42 (−46%; $P = 0.001$) (Figs. 2 and 3). A significant reduction of serum VEGF

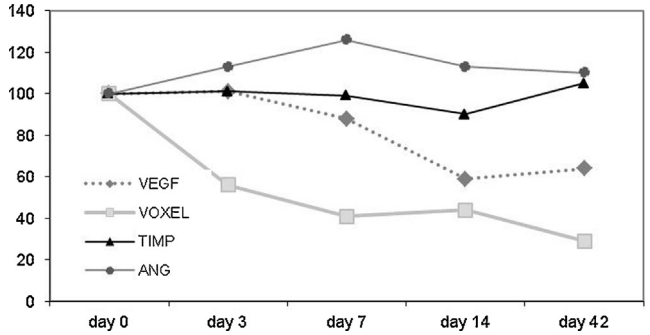

Fig. 2. Relative change of serum VEGF, angiogenin, TIMP 2 and Doppler activity (voxels) in the GC group.

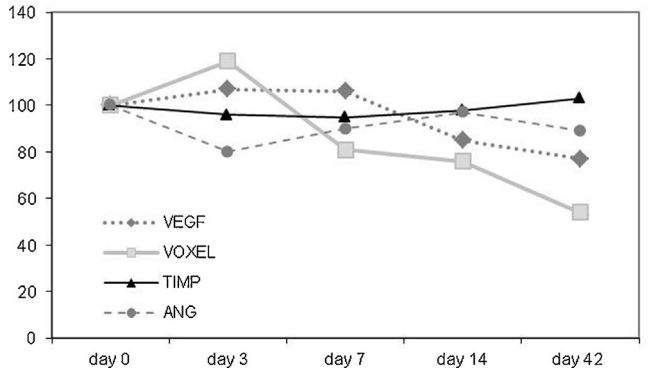

Fig. 3. Relative change of serum VEGF, angiogenin, TIMP 2 and Doppler activity (voxels) in the TNF group.

levels could be determined with a delay of 1 week after the decrease of Doppler activity in both treatment groups, respectively. The difference between the decrease of the Doppler activity at day 7 (−43%; $P = 0.003$) compared to the decrease of the serum VEGF levels (−5%; $P = 0.05$) was statistically significant ($P = 0.005$) (Fig. 4).

Whilst the amount of Doppler voxels correlated with the disease activity score (DAS 28) (rho: $P = 0.001$; r = 0.36), no correlation was found between serum VEGF and Doppler activity (rho: $P = 0.7$; r = −0.03). In addition, no correlation was found between serum VEGF levels and DAS 28 (rho: $P = 0.13$; r = 0.17).

Angiogenin and TIMP-2 levels did not change significantly in both groups. Angiogenin concentrations decreased initially in the TNF-α group and showed a slight increase in the GC group (Figs. 2 and 3).

4. Discussion

In RA inflammation, angiogenesis is a process that includes the accumulation of inflammatory cells and mediators as well as the invasive phenotype of the pannus at the cartilage and bone junction. Different processes promote angiogenesis in the synovia, one of them being TNF. Therefore, inhibition of TNF-α is a key treatment option to reduce angiogenesis. In 1999, Maini et al. enumerated the number of blood vessels in microscopic sections of synovial biopsy specimen. Their results indicated that the number of newly formed blood vessels was reduced after infliximab treatment [27]. In our own PDUS data, we could show indirectly with the aid of a three-dimensional blood vessel count that the number of intra-articular blood vessels was significantly reduced during 3 months of treatment with TNF-α inhibiting therapy [28].

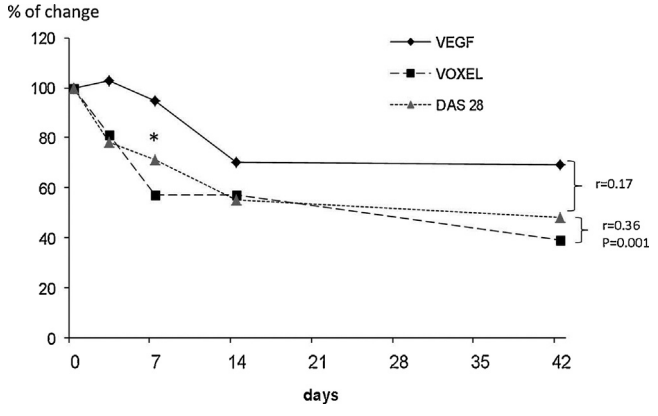

Fig. 4. Correlation of serum VEGF levels with Doppler activity (voxels) and DAS 28.
* $P = 0.005$ difference of decrease at day 7 between VEGF and voxels.

Data of small studies provided evidence that serum VEGF concentrations are higher in patients with early RA than in patients with longstanding treated RA [8,19,20]. This was consistent with our results and provides a common baseline for interpretation of the data.

With respect to the relation of VEGF to TNF-α inhibiting treatment, data from the study of Maini et al. showed that serum VEGF levels decreased significantly 1 week after infusion of infliximab [27]. The authors concluded that the reduction of angiogenesis is a result of the TNF-α regulation of endothelial growth factors by the reduction of serum VEGF. But it remained unclear which process triggers the cascade of events resulting in angiogenic inhibition, especially whether the reduction of VEGF is a specific effect of TNF inhibition or the result of suppressed inflammatory activity?

In accordance with results from a pilot study, we observed a significant decrease of VEGF during both GC and TNF-α inhibiting therapy [21]. Similar to our previous findings, the reduction of serum VEGF levels could already be seen after 2 weeks. The extension of follow-up until week 6 revealed a further decrease of VEGF during TNF-inhibiting treatment, whereas this effect could not be observed in the GC group.

PDUS has been demonstrated to be a sensitive tool for assessing angiogenesis and, thus, disease activity. The good intra- and inter-observer reproducibility of PDUS, confirmed by our reported inter-observer agreement values, enables the use of PDUS as an inexpensive and non-invasive instrument for the visualization of the synovial perfusion [25]. It also allows a comparison of PDUS activity with the kinetics of serum vascular markers. For example, two groups correlated synovial and serum VEGF concentrations, but results were contradictory [29,30]. Besides a positive correlation between the serum and synovial VEGF levels, Vreju et al. also provided evidence for a positive correlation of both parameters to the PDUS activity. As the synovial biopsy remains an invasive method, our approach was to use the PDUS method as an alternative imaging modality to reflect the dynamics of local angiogenesis in the synovia. In this context, we observed an interesting delay of decrease of the serum VEGF levels as compared to the immediate reduction of vascular flow demonstrated by PDUS.

Although PDUS activity correlated with clinical findings (DAS 28), there was no correlation with serum VEGF concentrations. Furthermore, in contrast to the data available in the literature, serum VEGF levels in our study did not correlate with scores of disease activity [20]. One explanation for this could be the different kinetics in the response of both parameters. Taken together, this is the first longitudinal set of data, in which information about the chronology of alteration in vascularization during treatment is provided especially as literature refers mainly to cross-sectional studies [22–24,29]. Our results suggest also that a therapeutic suppression of serum VEGF levels is not the adequate approach to reduce synovial perfusion as the decline appears beyond the vascular response. This idea is supported by the hitherto ineffective anti-VEGF trials [5,31].

In contrast to elevated angiogenin concentrations in synovial fluid in inflamed joints, plasma angiogenin concentrations have been found to be similar to healthy subjects and patients with osteoarthritis [11]. In our study, angiogenin was also only slightly elevated in the group of patients with longstanding RA. The kinetics of angiogenin was not significantly different in both therapy groups, although we observed an initial decrease in the TNF group and an increase in the GC group. These findings are less prominent than the change in VEGF levels and support also the hypothesis of a rather local role of angiogenin in the inflamed joint.

While data exists that MMP-1 and MMP-2 in their inactive form are reduced after infliximab treatment [27], there is no information about TIMP-2 concentrations during GC or TNF-inhibiting therapy. In our study, TIMP-2 levels where only slightly elevated in the

longstanding RA group and did not show a decrease during GC or anti-TNF therapy. These changes might contribute to the imbalance of promoters and inhibitors of angiogenesis in the process of RA inflammation [32] but their significance have to be interpreted with care with respect to the small patient collective.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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