

Quantification of VEGF and Screening of Transcription Factor HIF-1 α in Synovial Fluid of Polyarthritic Patients: Targets for Potential Anti-angiogenic Molecules

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Abstract

Rheumatoid arthritis (RA) is a polyarticular inflammatory autoimmune disease and osteoarthritis (OA) is an inflammatory degenerative disease of the articular cartilage. The current study efforts have been made to understand the relationship between the angiogenic genes VEGF, its regulator HIF-1 α and MVD in RA and OA. The level of VEGF in serum and synovial fluid was increased in both RA and OA, but the levels were slightly more in cases of RA compared to that of OA. A corresponding increase in expression of HIF-1 α and MVD counts was also noted in both conditions. Similar to VEGF levels, the expression of HIF-1 α , MVD and CD-31 counts was higher among RA patients. These results thus indicate that there is a significant association between the angiogenic peptide VEGF in serum and synovial fluids, expression levels of transcription factor HIF-1 α and microvessel density in the synovium of polyarthritic patients. The increased expression and regulation of VEGF, HIF-1 α and MVD in these chronic inflammatory conditions are not only early indicators but also potential targets for antiangiogenic factors in the therapeutics of novel drugs for these smoldering conditions.

Keywords: Rheumatoid arthritis; Osteoarthritis; Vascular endothelial growth factor; Hypoxia-inducible factor 1-alpha; Micro vessel density

Introduction

Rheumatoid arthritis (RA) is a polyarticular inflammatory autoimmune disease [1] and osteoarthritis (OA) is an inflammatory degenerative disease of the articular cartilage [2]. Inflammation and neoangiogenesis, an increased tendency for new blood vessel formation [3-6], is important in the development of new cartilage and mineralization in OA [7], whereas the same processes contributes for synovitis, pannus formation and articular cartilage destruction in RA [3]. Many of the pro-inflammatory proteins are responsible for disease progression in both the cases, which includes cytokines, chemokines, adhesion molecules and growth factors such as bFGF (Basic fibroblast growth factor) and VEGF [8].

High expression of VEGF, one of the key mediators of angiogenesis, as a consequence of increased micro vessel density (MVD) of the entire synovial vasculature, is seen in both RA and OA. However presence of an activated synovial vasculature (VEGF/KDR complex expression) was higher in cases of RA [9]. This is because; in OA there is failure of activation of this pathway in the presence of increased VEGF expression. Several studies indicated that VEGF also plays a role as proinflammatory protein and expression of this protein is measured in inflammatory fluids including synovial fluid from RA and OA [8,10,11]. VEGF gene is regulated by hypoxia and is controlled by the transcription factor HIF-1 α . Reduction in the degradation rate of HIF-1 α under hypoxic stress results in accumulation of HIF-1 α

proteins and up regulation of angiogenic process [12,13]. The direct link between accumulation of HIF-1 α and over expression of VEGF reflects its importance in inducing angiogenesis in arthritis, suggesting the key role of HIF-1 α in the pathogenesis of RA and OA [14,15].

Multiple proinflammatory signaling systems with redundant effects on angiogenesis are known to operate in RA and other inflammatory diseases of the joint [16,17]. Hence the purpose of the present study was to investigate the association of angiogenic gene VEGF and its regulator HIF-1 α to evaluate their relationship with RA and OA. By ELISA and immunohistochemical studies we found the expression level of HIF-1 α , VEGF with increased MVD more in RA than in OA.

Materials and Methods

Patients and controls

Patient's recruitment and sample collection was done from the laboratory personnel and the donor service of J.S.S. Hospital Mysore, India. It was performed according to the guidelines and protocols approved by the institutional review boards. Informed consent was obtained from all subjects. Diagnosis of RA and OA was done based on conventional clinical and supporting laboratory parameters by the clinicians. Inflammatory synovial fluid and serum from polyarthritic patients was collected in sterile tubes from the department of orthopedics. The study included 133 patients diagnosed with RA and 122 patients diagnosed with OA. The samples were centrifuged at 10,000 g for 10 min at 4°C. The cell free supernatant was collected and the aliquots were stored and frozen at -80°C. The cell pellet was examined microscopically to note the cytological details. Synovial

biopsy of 25 patients each from RA and OA were fixed in 10% formalin and embedded in paraffin.

Proteins and antibodies

Human recombinant VEGF165 was introduced into PET3 vector and transformed into *E. coli* cells. The VEGF production was induced and collected from cell pellets as explained earlier [18]. Polyclonal affinity antibodies purified from rabbit antisera against these proteins were prepared as reported previously [19]. To state briefly, rabbits were immunized with purified human proteins, total IgG from rabbit serum was isolated using antigen coupled sepharose columns. Monospecificity of rabbit anti-human Rh VEGF165 antibody was analyzed by immunoreactivity against purified proteins and by western blot analysis. No cross reactivity against the homologous protein was detected.

Sandwich ELISA

Concentration of VEGF was determined using a double sandwich enzyme linked immunosorbent assay (ELISA) system established at the department of Applied Botany and Biotechnology, Manasgangothri, Mysore. Flat bottom 96 well microtitre plates (Maxisorp, Nunc, Hamburg, Germany) were coated with 100 μ L/well of 10 μ g/mL captured antibody in 0.1 M sodium carbonate buffer at pH 9.6. It was incubated overnight at 4°C, washed three times with phosphate buffered saline and 0.1% tween 20 at pH 7.4 (wash buffer) and blocked with wash buffer containing 0.25% Bovine serum albumin (block buffer) for 1 h at 37°C.

After washing again with wash buffer, 100 μ L of samples in varying dilutions with blocking buffer was added and kept for 2 h at room temperature. The ELISA was calibrated with purified proteins in concentration ranging from 50 pg/mL to 5 ng/mL. The assay has a linear range between 0.005 and 5 ng/mL and sensitivity of <50 pg/mL. After three washes, HRP conjugated detector antibody 10 μ g/mL was added and incubated further for 1 h at 37°C. Again after washing three times, the plates were incubated with OPD substrate for 20 min at room temperature. Absorbance at 490 nm was measured using an automatic ELISA reader.

Immunohistochemistry

Five micron sections of synovial tissue of arthritic patients fixed in formalin and were embedded in paraffin. The sections were immunostained for CD31 and HIF-1 α as per the protocol supplied by the manufacturer (Santa Cruz Biotechnology, CA, USA). In brief, sections were de-paraffinized by using xylene and hydrated with 100% ethanol for 5 min, followed by 95% ethanol for 2 min, 80% ethanol for 2 min and then by immersing in subsequent graded alcohol the sections were brought to water. The hydrated sections were incubated with 3% H₂O₂ in PBS to block endogenous peroxidase activity. After 2 min, the sections were rinsed with PBS for 2 min, baked at 450 Hz for 10 min to retrieve the antigen, and hydrated with PBS for 2 min. Sections were incubated with anti CD31 (PECAM-1) and anti-HIF-1 α antibodies overnight at 4°C.

After tapping off the antibody, the slides were dipped for 5 min in PBS. The sections were incubated with secondary antibody with biotinylated rabbit anti-mouse IgG for 30 min at room temperature. The slides were washed in PBS for 5 min and incubated with ABC-reagent for 45 min, followed by histobuffer wash and subsequent incubation with substrate DAB peroxidase for 10 min. The sections

were then counterstained with 2% hematoxylin solution for 5 min and washed in water. The slides were further dehydrated and mounted using DPX. Appropriate positive and negative controls were included with each set of stains. One section from each specimen was routinely stained with H & E. Microvessel density count was derived by counting the average number of stained vessels per ten high powered fields using LietzDiaplan bright field microscope attached to camera.

Statistical analysis

Statistical analysis was performed with the SPSS for windows program package. Data were expressed in the mean \pm standard deviation (S.D.). P values of 0.05 were considered statistically significant.

Results

Out of 255 patients included in the study, 133 patients were diagnosed with Rheumatoid arthritis (RA) and the remaining 122 were diagnosed with Osteoarthritis (OA). The average age of the patients with RA was 51.35 with the range between 16 to 89 years while the average age of patients with OA was 46.57 years with the range between 14 to 76 years (Table 1).

Patient's characteristics		
Patients	RA	OA
Average age (Years)	51.35 (16-89)	46.57 (14-76)
Gender		
Male	61	62
Female	72	60

Table 1: Details of patients included in the study with respect to age and gender.

The patients were categorized based on the age, gender and the clinical criteria in which positive for the presence of Rheumatoid Factor in synovial fluid was considered (Table 2).

Cytological features		
Type of arthritis	RA	OA
Average cell count	460-1500	800-4500
Predominant cell type	Lymphocytes	Neutrophils

Table 2: Details of cytological features among RA and OA patients.

The cytological details of the synovial fluids showed a predominance of lymphocytes in RA and neutrophils in OA with a cell count of 460 to 1500 in RA and 800 to 4500 in OA.

VEGF in sera and effusions of patients with RA and OA

Measurement of angiogenic growth factor VEGF was carried out by developed sandwich ELISA using antigen affinity purified anti VEGF165 polyclonal antibodies. The level of VEGF in serum of normal healthy subjects (n=10) was low with a mean of 30 pg/ml (range L: 5-90). Patients with RA showed a mean VEGF levels of 147.22 pg/ml (range: 60-280 pg/ml) in serum and 146.66 ng/ml (range:

65-210) in synovial fluid. In cases of OA, the mean VEGF levels were 94.33 pg/ml (range: 40-230 pg/ml) and 90.00 ng/ml (range 25-175 ng/ml) in serum and synovial fluid respectively. There was a significant difference observed in both the serum and synovial fluid VEGF levels between RA and OA, with higher levels being noted in both serum and synovial fluid of RA patients compared to OA patients (Table 3).

Clinical condition	No. of patients	Concentration of VEGF		
		Synovial fluid Ng.ml ⁻¹	Serum Ng.ml ⁻¹	P value
Rheumatoid arthritis	133	145.66	0.1472	0.0001*
Osteoarthritis	122	90.66	0.0943	0.0001*
Health individual	10	ND	0.03	ND
ND: not determined				

Table 3: Level of VEGF in various polyarthrititis conditions.

MVD/CD-31 and HIF-1 α expression in tissues of OA and RA

Synovial tissue from 25 cases each of RA and OA were evaluated for micro vessel density (MVD) and expression of endothelial marker CD-31.82% of the cases showed an increased MVD and positive immunoreactivity towards endothelial marker CD-31 (Figure 1). The mean MVD in the synovial sections of RA patients was 25/HPF (range: 14-35) whereas in OA the mean MVD was 17/HPF (range 8-26) (Table 4).

To study the influence of the transcription factor HIF-1 α expression in both RA and OA immunohistochemical staining was performed using anti-HIF-1 α antibodies. Expression of HIF -1 α was seen both within the cytoplasm and the nucleus of the endothelial cells in the synovial tissue (Figure 2). The mean percent of cells with positive HIF-1 α expression examined in the case of RA patients was 78% whereas in case of OA it was 68% (Table 4).

Samples	No. of patients	MVD/HPF	HIF-1 α % expression in Synovium
Rheumatoid arthritis	133	25 (14-35)	78%
Osteoarthritis	122	17 (8-26)	68%
Health individual	10	ND	ND
ND: not determined			

Table 4: Expression levels of MVC and HIF-1 α in various polyarthrititis conditions.

Relationship between VEGF, HIF-1 α and MVD in RA and OA

The above results were evaluated to study the relationship between the angiogenic genes VEGF, its regulator HIF-1 α and MVD in RA and OA. The level of VEGF in serum and synovial fluid was increased in both RA and OA, but the levels were slightly more in cases of RA compared to that of OA. A corresponding increase in expression of HIF-1 α and MVD counts were also noted in both conditions.

Similar to VEGF levels, the expression of HIF-1 α , MVD and CD-31 counts were higher among RA patients. These results thus indicate that there is a significant association between the angiogenic peptide VEGF in serum and synovial fluids, expression levels of transcription factor HIF-1 α and microvessel density in the synovium of polyarthritic patients. The increased expression and regulation of VEGF, HIF-1 α and MVD in these chronic inflammatory conditions are not only early indicators but also potential targets for an effective role of anti angiogenic factors in the therapeutics of novel drugs for these smoldering conditions (Figures 1 and 2).

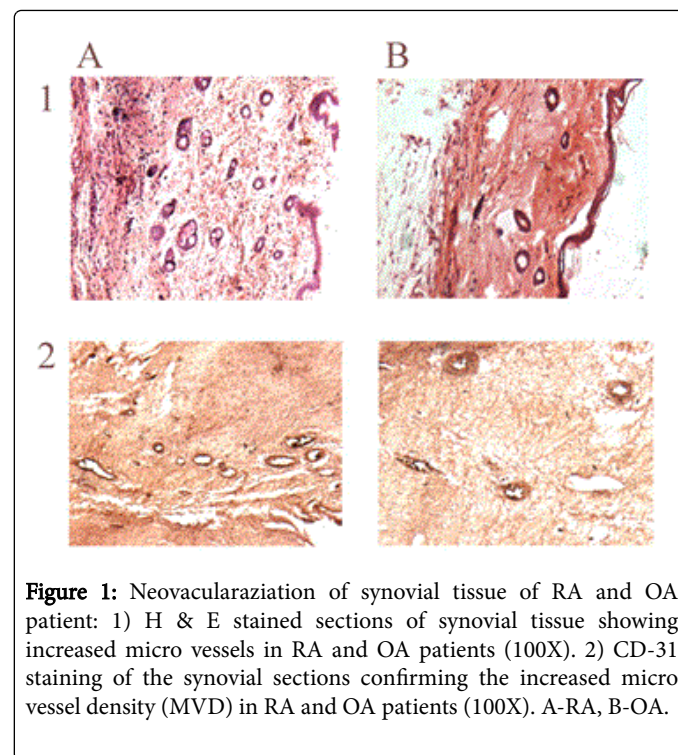


Figure 1: Neovascularization of synovial tissue of RA and OA patient: 1) H & E stained sections of synovial tissue showing increased micro vessels in RA and OA patients (100X). 2) CD-31 staining of the synovial sections confirming the increased micro vessel density (MVD) in RA and OA patients (100X). A-RA, B-OA.

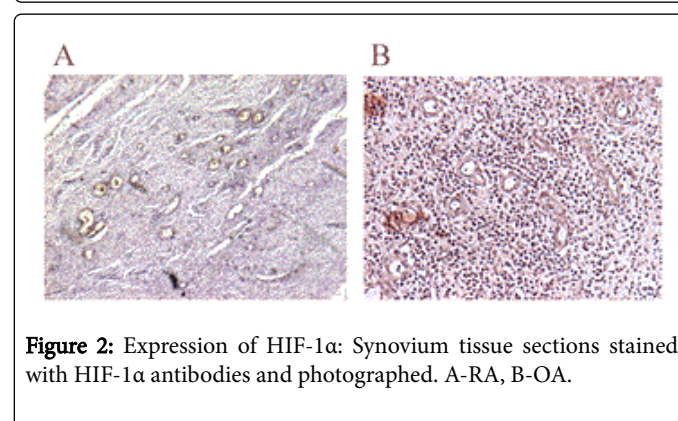


Figure 2: Expression of HIF-1 α : Synovium tissue sections stained with HIF-1 α antibodies and photographed. A-RA, B-OA.

Discussion

Angiogenesis and inflammation are codependent processes in arthritic conditions [20]. Chronic inflammation can stimulate vessel growth and this potentiates tissue altered inflammatory response. RA and OA are inflammatory angiogenic disorders caused by involvement of several mediators and cells such as monocytes, macrophages, fibroblasts and T-cells. These release numerous cytokines as well as VEGF, which can be detected in sera and synovial fluid. The role of

these cytokines in pathogenesis of these diseases has been widely reported [8,21,22]. However, very little is known about the role of HIF in RA and OA pathology and its importance in angiogenesis. So far no reports are available which correlates the expression of angiogenic peptides VEGF, HIF and MVDs in both OA and RA [8].

VEGF is a potent angiogenic factor, which enhances the microvascular permeability by direct action on vascular endothelium and fibroblasts. During arthritic conditions, angiogenesis increases with increase in expression of VEGF [8,23]. The expression of VEGF level in synovial fluid and tissue correlated with clinical severity of human RA and OA and degree of joint destruction [24,25]. The synthesis of VEGF by inflammatory and synovial cells is induced by hypoxia via HIF-1 α [26]. The elevated VEGF levels in serum and effusions in different inflammatory disorders have also been reported previously and the same has been noted in the present study. HIF-1 α expression was more prominent in RA and there was a significant correlation with HIF-1 α , VEGF and MVD [9] as in the present study. Up-regulation of HIF-1 α in the osteoarthritic synovium is also associated with increased microvascular density and expression of angiogenic factors, indicating that hypoxia may play an additional mediating role in OA. The lack of HIF-1 α expression by normal synovium has also been reported [27]. Our studies showed higher expression levels of VEGF in sera and effusions of RA than in OA. This is similar to earlier reports that synovial fluid and serum levels of the VEGF may be higher in groups of patients with RA compared with OA [10,11]. Some authors have, however, reported that OA patients can display lower angiogenic potential than patients with RA [8]. We observed similar difference in angiogenic potential between RA and OA with respect to decrease in microvessel count and HIF-1 α expression which are directly associated to one another [9]. We also found a significant association of VEGF expression, microvessel density and HIF-1 α expression. Based on the above facts we further ascertain that angiogenesis plays an important role in both RA and OA.

Conclusion

The detection of VEGF in inflammatory fluids is extremely important to understand the role of this proteins in angiogenic inflammatory disorders. This information is validated in homogenous, large scale perspective studies which may be clinically relevant in terms of (a) avoiding unnecessary chemotherapy or hormone therapy in patients with high levels of VEGF, which indicates that the underlying disorder is an angiogenic inflammatory disorder (b) VEGF can serve as targets for therapy and be potentially useful predictive markers for novel therapeutic strategies with anti-VEGF/anti-angiogenic compounds that can inhibit angiogenesis and inflammation and further (c) VEGF receptors seem to be ideal candidate as biomarkers for inflammation in human RA and OA. This can be employed for evaluation of the biological activity of anti-inflammatory and anti-angiogenic agents in early clinical studies.

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