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Upregulation of stromal cell derived factor-1α in collagen vascular diseases-associated interstitial pneumonias (CVDs-IPs)

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ABSTRACT

Objective: We speculated that distinct angiogenic profiles are involved in idiopathic interstitial pneumonias (IIPs) in comparison with interstitial pneumonias associated with collagen vascular disease (CVD-IPs). This hypothesis was investigated by measuring the expression of a cardinal biologic axis, the vascular endothelial growth factor (VEGF)–stromal derived growth factor [SDF-1α, transcripts 1 and 2 (TR1 and TR2)] and receptor, CXCR4 and the angiogenetic receptors CXCR2 and CXCR3 in bronchoalveolar lavage fluid (BALF) in both conditions.

Methods: We studied prospectively 25 patients with fibrotic IIPs (f-IIPs) [20 with idiopathic pulmonary fibrosis (IPF) and 5 with idiopathic non-specific interstitial pneumonia (NSIP)] and 16 patients with CVD-IPs. mRNA expression was measured by Real-Time RT-PCR and protein was evaluated by Western Blotting.

Results: A significantly greater value has been detected in SDF-1α-TR1 mRNA expression levels of CVD-IPs ($p = 0.05$) in comparison with IPF group. A similar trend has been also detected in protein expression in favor of CVD-IP group. In addition, VEGF mRNA levels have been found significantly increased in CVD-IPs in comparison with the NSIP group ($p = 0.05$). No significant difference has been found in SDF-1α-TR2–CXCR4 mRNA and CXCR2–CXCR3 between the two groups.

Conclusion: These results showed increased expression of SDF-1α in CVD-IPs, suggesting different angiogenic procedures. Further studies are needed in order to better explore the angiogenetic pathway in these disorders.

1. Introduction

Connective tissue disorders (CTDs) represent a heterogeneous group of immunologically mediated inflammatory disorders with a large variety of affected organs. Interstitial pneumonia (IP) is frequent in CTDs with an overall incidence estimated at 15% [1]. It has been reported that patients with CTD-IP have a better prognosis than patients with idiopathic interstitial pneumonia (IIP) [2–6], and that was primarily related to a higher frequency of non-specific interstitial pneumonia (NSIP) in patients with CTDs. It has been recently observed that even patients with CTD–usual interstitial pneumonia pattern (UIP) have better prognosis than patients with idiopathic pulmonary fibrosis (IPF), despite the same histologic pattern [7].

The underlying pathogenesis of fibrosis in these diseases may be distinctly different, however, poorly investigated in the current literature. It has been previously demonstrated that there is a lower profusion of fibroblastic foci in patients with CTD–UIP than in patients with IPF–UIP, despite a similar amount of fibrosis on HRCT [3]. Recent theories implicate recurrent injurious exposure, imbalance that shifts Th1/Th2 equilibrium towards Th2 response and angiogenesis in the pathogenesis of pulmonary fibrosis [8].

The concept of vascular remodeling in IPF was originally identified by Turner-Warwick [9]. In more recent morphologic reports, Renzoni et al. have also observed vascular remodeling in both IPF and fibrosing alveolitis associated with systemic sclerosis [10]. Moreover, Cosgrove and coworkers provided further support of vascular remodeling in IPF demonstrating decreased vascular density within the fibroblastic foci [11]. Furthermore, our

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department has demonstrated a distinct profile of angiogenic and angiostatic chemokines in IPF and sarcoidosis patients that may explain differences between these two diseases in terms of pathogenetic mechanisms [12].

It is of note that vascular endothelial growth factor (VEGF) is a potent angiogenic factor. Stromal derived factor 1 (SDF-1, CXCL12) is a CXC chemokine which despite the lack of ELR motif has an angiogenic activity, and its receptor is CXCR4. It has been demonstrated that there is a positive feedback loop of VEGF inducing CXCR4 and CXCL12 expression by endothelial cells and conversely CXCL12 interaction with CXCR4 enhances VEGF expression of these cells [13–15]. Furthermore, known ligands for CXCR3 are three angiostatic ELR^- CXC chemokines (Mig/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11) [9–12]. We have shown that sarcoidosis exhibits a distinct angiostatic profile, as shown by an ELR^- CXC chemokine upregulation in comparison to IPF patients [12]. On the other hand, ELR^+ CXC chemokines (IL-8, ENA-78 and GRO-α) sharing the same receptor, CXCR2, were found increased in IPF [12], while a down-regulation of their levels was recently shown after treatment [13].

The purpose of our study was to assess levels of these angiogenic factors in the BALF of patients with f-IIPs (IPF and NSIP) and CVD-IPs in order to test the hypothesis that a distinct angiogenic profile between these two diseases could partially explain the differences in terms of pathogenetic mechanisms and different prognosis.

2. Patients and methods

2.1. Patients

From April 2006 to January 2008, forty-one [41] consecutive patients from the Interstitial Lung Disease Unit of the Department of Thoracic Medicine, University Hospital of Heraklion were enrolled in the study: 25 patients with idiopathic fibrotic pneumonias (f-IIPs) and 16 patients with fibrosis related to CVDs (CVD-IPs).

First group (f-IIPs): The diagnosis was based on internationally accepted clinical and imaging criteria [16]. 20 patients were characterized as IPF and 5 as NSIP. The diagnosis of IPF was made in 7 cases by video-assisted thoracoscopic surgery (VATS) biopsy (in the correct clinical context, detailed below); the histologic diagnosis of usual interstitial pneumonia (UIP) was obtained in six cases, while the pattern of NSIP was found in one patient. In the remaining 18 cases the diagnosis was made on the basis of clinical and high-resolution computed tomography (HRCT) criteria: (1) bilateral basal or widespread crackles; (2) restrictive ventilatory defect or isolated depression of DL_{CO}; (3) computed tomography (CT) appearances indicative of IPF with predominantly basal and subpleural microcystic or macrocystic honeycombing, with variably extensive ground-glass and reticular abnormalities but no consolidation, nodular abnormalities, or other parenchymal abnormalities (apart from centrilobular emphysema); and (4) no environmental exposure to a fibrogenic agent or connective tissue disease [16]. According to the aforementioned criteria a known cause of pulmonary fibrosis, such as a connective tissue disorder, has been excluded by both immunologic screening and rheumatological clinical evaluation.

Second group (CVD-IPs): The diagnosis was based on clinical and HRCT criteria in accordance with the international societies [7,17,18]. In detail, a. 7 patients with rheumatoid arthritis (RA): Four with HRCT characteristics of UIP, 1 with NSIP and two with imaging features of organizing pneumonia (OP), b. 4 patients with systemic sclerosis (SSc) and HRCT appearance of NSIP, c. 2 patients with systemic lupus erythematous (SLE) and HRCT features of NSIP, d. one patient with Sjogren syndrome and histologically proven fibrotic NSIP, e. one patient with dermatomyositis–polymyositis and HRCT features of NSIP, f. one patient with mixed connective tissue disease and NSIP imaging on HRCT. Thus, the second group consisted of 4 UIP, 10 NSIP and two patients with OP.

2.2. Methods

2.2.1. Pulmonary function tests

All patients were evaluated spirometrically and by measurement of lung volumes, diffusion capacity and arterial blood gases (at rest). Spirometry and lung volumes (helium-dilution technique) and $T_{L,CO}$ (corrected for the haemoglobin) using the single-breath method were performed by a computerised system (Jaeger 2.12; MasterLab, Würzburg, Germany). Predicted values were obtained from the standardised lung function testing of the European Coal and Steel Community, Luxembourg (1993) [19]. Arterial blood gases were measured by an arterial blood gas analyser (AVL330; MasterLab system).

2.2.2. BAL fluid processing

BALF was obtained from patients with f-IIPs and CVD-IPs by methods previously described [12,13]. Briefly, a flexible bronchoscope was wedged into a subsegmental bronchus of a predetermined region of interest based on radiographical findings. A BAL was performed by instilling a total of 240 mL of normal saline in 60-mL aliquots, each retrieved by low suction. The BALF fractions were pooled and split equally into two samples. One sample was sent to the clinical microbiology and cytology laboratory and the other sample was placed on ice and transported to the research laboratory. The research sample was filtered through sterile gauze (Thompson, Ontario, Canada) and centrifuged at 400*g* for 15 min at 4 °C.

Total cell counts were determined using an improved Neubauer counting chamber, and expressed as the total number of cells per mL of aspirated fluid. The pellet was washed three times with cold PBS-Dulbecco's and the cells were adjusted to a final concentration of 10^6 cells/mL with RPMI1640 plus 2% FCS. The slide preparation was performed as previously reported [20]. The authorization has been achieved from the Ethical Committee of the University General Hospital of Heraklion.

2.2.3. RNA isolation and reverse transcription-polymerase chain reaction

2.2.3.1. RNA extraction and reverse transcription. Total RNA was extracted from each specimen using a power homogenizer and the TRIzol® reagent (Invitrogen, Carlsband, CA) according to the manufacturer's instructions. cDNA was synthesized using the Strascript reverse transcriptase kit (Stratagene, La Jolla, CA) as previously described [21].

2.2.4. Real-time RT-PCR

Peptide growth factors mRNA expression was measured using a real-time RT-PCR assay with SYBR-Green I. Primers were designed to span introns [21]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, in order to normalize VEGFA, SDF-1α or CXCL12, transcripts 1 and 2 CXCR4 and CXCR2, CXCR3 expression levels (Table 1). Specifically, 1 μl cDNA from pathological or control samples was amplified in a PCR reaction containing 2X Brilliant SYBR-Green I QPCR Master Mix, 300 nM of each primer and 30 μM ROX passive reference dye, in a final volume of 20 μl. After an initial denaturation at 95 °C for 10 min, the samples were subjected to 40 cycles of amplification, comprised of denaturation at 95 °C for 30 s, annealing at appropriate temperature for each primer pair for 30 s and elongation at 72 °C for 30 s, followed by a melt curve analysis, in which the temperature was increased from 55 °C to 95 °C at a linear rate of 0.2 °C/s. Data