Lymphatic endothelial progenitor cells and vascular endothelial growth factor-C in spondyloarthritis and Crohn's disease: two overlapping diseases?

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Abstract

Objective

The role of the lymphatic system in the connection between spondyloarthritis (SpA) and Crohn's disease (CD) remains yet to be elucidated. The aim of the present study was to investigate the circulating levels of lymphatic endothelial progenitor cells (LEPCs) and vascular endothelial growth factor-C (VEGF-C) and their possible correlation with clinical parameters in SpA, SpA associated with CD (SC), and CD.

Methods

Peripheral blood samples from SpA (n=36), SC (n=20) and CD (n=28) patients and 20 age- and sex-matched healthy controls were collected and used for quantification of circulating LEPCs and VEGF-C. LEPCs were identified by fluorescence-activated cell sorting using FITC-CD34, APC-CD133 and PE-VEGFR-3 antibodies. Serum levels of VEGF-C were measured by enzyme-linked immunosorbent assay. The possible correlations between disease duration (< or >10 years; < or >20 years) and clinical activity (BASDAI for SpA or CDAI for CD) and LEPC counts and VEGF-C levels were analysed.

Results

Circulating LEPC levels were significantly increased in SpA (p=0.0006) and SC (p=0.0058) patients compared with controls. In CD patients, LEPC counts negatively correlated with disease duration, with lower levels in longstanding disease (>20 years, p=0.018), but were not different from controls. No significant difference in VEGF-C levels was found in SpA, SC and CD compared with controls. Both LEPC and VEGF-C levels were independent of BASDAI and CDAI.

Conclusion

On the basis of our observations, an active mobilisation of lymphatic endothelial cell precursors was observed only for spondylitis involvement.

Key words

spondyloarthritis, Crohn's disease, lymphatic endothelial progenitor cells, VEGF-C

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Introduction

In the last decades the link between joint and gut inflammation has been widely investigated. In fact, spondyloarthritis (SpA) might be frequently associated with extra-articular manifestations, such as microscopic subclinical gut inflammation that is an important risk factor for Crohn's disease (CD) evolution (1-5). On the other hand, CD is a chronic inflammatory gastrointestinal disease that might also have subclinical articular SpA involvement (6, 7).

It is widely accepted that a combination of factors such as genetics, immunity, infections and environmental factors may be involved in SpA and CD etiopathogenesis (8, 9). In particular, it is known that the genetic susceptibility may overlap in both diseases (1). Lymphatic vessels have an important role both in acute and chronic inflammation with controversial protective or harmful effects as well. Lymphatic endothelial cells are fully differentiated cells that need an appropriate microenvironment to retain their functions and features (10) whereby they are conditioned by inflammatory processes but, reciprocally, might also influence flogosis (11). Inflammation induces the production of growth factors for lymphatic vessels, such as vascular endothelial growth factor-C (VEGF-C), and leads to intense de novo lymphangiogenesis, lymphovasculogenesis and lymphatic vessel remodeling (12-15). Furthermore, recent growing evidence shows that bone marrow-derived lymphatic endothelial progenitor cells (LEPCs) may contribute to postnatal lymphangiogenesis and lymphovasculogenesis (14, 15). The main biomarkers of LEPCs are CD34, CD133 and VEGF receptor-3 (VEGFR-3 or Flt4), and under induction with VEGF-C these cells can differentiate into lymphatic endothelial cells via VEGFR-3 signaling (14). In addition, it has been reported that during inflammatory conditions, tissue macrophages may also contribute to lymphangiogenesis via their differentiation into lymphatic endothelial cells that integrate into sprouting lymphatic vessels (16). Besides lymphangiogenesis, an important aspect of the functional impairment of lymphatic vessels, occurring during inflammatory processes, might be the abnormal accrual of lymph fluid in the tissue that culminates in lymphoedema (15). Although lymphangiogenesis and lymphoedema have been noticed and described both in SpA (17-18) and in CD (19-20), these aspects remain yet to be deeply elucidated.

On these premises, the aim of the present study was to evaluate the circulating levels of LEPCs and VEGF-C in patients with SpA compared with SpA associated with CD (SC), CD without skeletal involvement and healthy subjects. Moreover, we also analysed the possible correlation between LEPC and VEGF-C levels, disease duration and clinical activity.

Materials and methods

Study subjects

Thirty-six patients with axial SpA diagnosed with ASAS criteria, twenty-eight patients with CD and twenty patients with SpA associated with CD (SC) were enrolled at the Rheumatology Unit, Digestive Surgery Unit and General, Emergency and Mininvasive Surgery I Unit, Azienda Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy. All participants gave written informed consent and the study was approved by the institutional review board. From each of these patients and from twenty age-matched and sex-matched healthy subjects, without inflammatory bowel and articular symptoms, two samples of whole peripheral blood were collected: one for mononuclear cell isolation (10ml) and the other one for serum separation (5ml). Exclusion criteria were age <18 years, cancer or presence of other inflammatory bowel diseases (coeliac syndrome, ulcerative colitis) or other rheumatic diseases (crystal-mediated arthritis, rheumatoid arthritis, connective tissue diseases).

Sample preparation

Mononuclear cells from peripheral blood samples were separated by density gradient centrifugation, following a standard protocol. Briefly, blood samples were diluted with phosphate-buffered saline (PBS), stratified on half volume of Lympholyte (Cedarlane Laboratories, Burlington, Ontario, Can-

ada) and centrifuged at 3000 rpm for 30 minutes at room temperature. After separation, white blood cells were recollected, washed with PBS and centrifuged at 1200 rpm for 5 minutes at room temperature. Subsequently, the pellet was treated with Red Cell Lysis Buffer to ensure red blood cell elimination and washed in PBS.
For serum collection, whole peripheral blood was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected, transferred into clean tubes and stored at -80°C until use.

Fluorescence-activated cell sorting (FACS) analysis
For FACS analysis, samples were incubated with three different fluorochrome-conjugated monoclonal antibodies (FITC-CD34, APC-CD133 and PE-VEGFR-3) recognising LEPC-specific antigens (21). According to literature (21), LEPCs were defined as CD34+/CD133+/VEGFR-3+ circulating cells. In particular, a 100µl aliquot of each sample was stained with 1µl each of FITC-CD34 (BD Biosciences, Franklin Lakes, NJ, USA), APC-CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) and PE-VEGFR-3 (R&D Systems, Minneapolis, MN, USA) and incubated in the dark in ice for 15 minutes. For each sample, a control tube with no antibodies was prepared. After washing cells with PBS, FACS analysis was performed using a FacsCanto (BD Biosciences, Franklin Lakes, NJ, USA). LEPC counts were reported as an absolute number per $2x10^5$ mononuclear cells and results were expressed as a percentage of total mononuclear cells.

Enzyme-linked immunosorbent assay (ELISA) for VEGF-C
Serum was isolated from whole blood as previously described and VEGF-C levels were evaluated by Human VEGF-C Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), following the manufacturer's protocol. Each sample was measured in duplicate.

Clinical parameters
For the patients the following clinical parameters were evaluated: BASDAI (0–10; active disease >4/10) (22), CDAI (<150 remittent; 150–250 low; 250–350 moderate; >350 severe), SpA and CD disease duration, SpA involvement (isolated axial involvement and/or association with peripheral or entheseal disease) and actual therapeutic treatments. Endoscopic Mayo score was also considered for CD and SC. Differences between patients with different clinical activity (BASDAI <4 *vs*. >4 and, on the basis of CDAI, remittent *vs*. low-moderate-severe CD activity), SpA different involvement (isolated axial disease *vs*. association with peripheral and entheseal disease), SpA and CD standing (duration <10 *vs*. >10 years, <20 *vs*. >20 years) were evaluated. Correlation with disease duration, BASDAI and CDAI was also analysed.

Statistical analysis
Descriptive statistics were expressed as mean ± standard error of the mean (SEM), 95% confidence interval (CI) and range for continuous variables, and as number and percentage for categorical variables. Normal distribution of parameters was verified by Kolmogorov-Smirnoff test. In particular, the statistical significance of the differences was evaluated by unpaired *t*-test with Welch correction and Mann-Whitney U-test. Correlation was evaluated with Pearson or Spearman test as appropriate. $p<0.05$ was considered statistically significant.

Results

Demographic and clinical data of healthy subjects and patients are shown in Table I. No difference of age was significant at Kruskall Wallis test. Sex and age distribution matched between different groups of patients and healthy controls. For both LEPC and VEGF-C levels, no significant correlation neither statistical difference were observed in relationship to age, sex and treatments. CDAI score of CD and SC patients was in accordance with endoscopic Mayo score.

Circulating levels of LEPCs in patients and healthy controls
LEPC levels were evaluated by FACS analysis in the different groups of subjects enrolled in the study. Representative scatter plots for each group are reported in Fig. 1A-D. As it can

Table I. Demographic and clinical data of patients and healthy subjects.

	SpA (n=36)	SC (n=20)	CD (n=28)	Healthy controls (n=20)
AGE (mean ± SD, range)	54 (±11.2) (33-71)	53 (±12) (34-80)	47 (±13) (28-69)	53 (±12) (30-78)
Sex	13 M: 23 F	7 M: 13 F	15 M: 13 F	9 M: 11 F
Duration of disease (years) (mean ± SD, range)	11.5 (±11.9) (1-40)	SpA: 9.5 (±9.1) (1-30) CD: 16,2 (10,8) (5-34)	12.8 (±17) (2-39)	NA
Peripheral and entheseal involvement	16/36 and 14/36	6/20 and 8/20	NA	NA
BASDAI (0-10) (mean ± SD, range)	4.3 (±2.63) (0-10)	4.96 (±31.1) (0-8.6)	NA	NA
CDAI (remission-low-moderate-severe)	NA	9/20 remittent; 4/20 low; 5/20 moderate; 2/20 severe	3/20 remittent; 7/20 low; 2/20 moderate; 16/20 severe	NA
Treatments	6/36 biologics and 10/36 DMARDs	4/20 biologics and 12/20 DMARDs	5/28 biologics and 11/28 DMARDs	NA

CD: Crohn's disease; SpA: spondyloarthritis; SC: spondyloarthritis associated with CD; N.A.: not applicable.

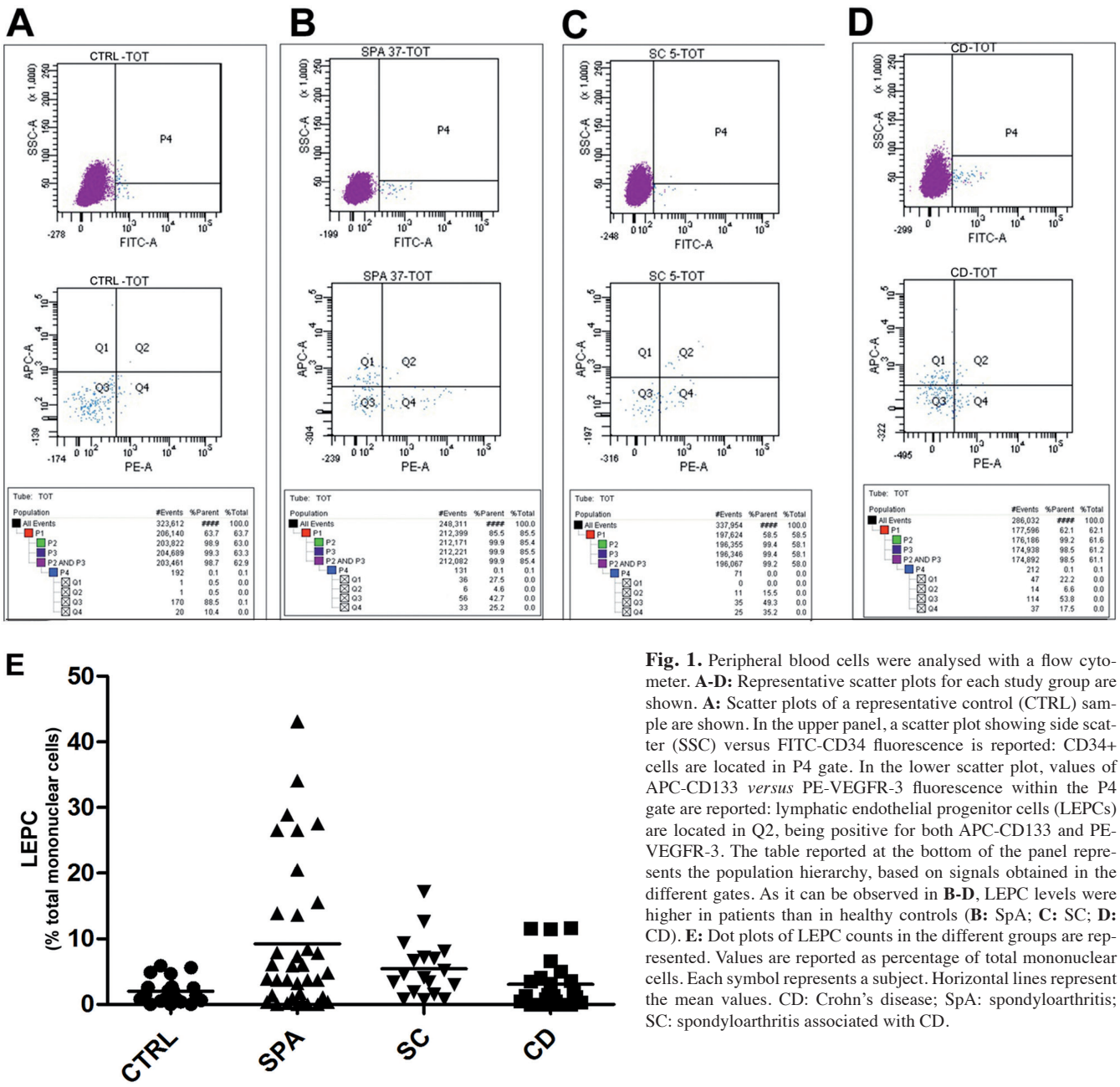

Fig. 1. Peripheral blood cells were analysed with a flow cytometer. **A-D:** Representative scatter plots for each study group are shown. **A:** Scatter plots of a representative control (CTRL) sample are shown. In the upper panel, a scatter plot showing side scatter (SSC) versus FITC-CD34 fluorescence is reported: CD34+ cells are located in P4 gate. In the lower scatter plot, values of APC-CD133 *versus* PE-VEGFR-3 fluorescence within the P4 gate are reported: lymphatic endothelial progenitor cells (LEPCs) are located in Q2, being positive for both APC-CD133 and PE-VEGFR-3. The table reported at the bottom of the panel represents the population hierarchy, based on signals obtained in the different gates. As it can be observed in **B-D**, LEPC levels were higher in patients than in healthy controls (**B:** SpA; **C:** SC; **D:** CD). **E:** Dot plots of LEPC counts in the different groups are represented. Values are reported as percentage of total mononuclear cells. Each symbol represents a subject. Horizontal lines represent the mean values. CD: Crohn's disease; SpA: spondyloarthritis; SC: spondyloarthritis associated with CD.

be observed in Fig. 1E, LEPC levels were higher in patients than in healthy controls. In particular, statistically significant differences emerged when comparing healthy subjects with patients affected by SpA (p=0.0006) and SC (p=0.0058) (Fig. 1E), while there was no significant difference between healthy subjects and CD. LEPC counts were higher in both SpA and SC than in CD, but these differences did not reach statistical significance. Results on LEPC counts reported as percentage of total mononuclear cells are summarised in Table II.

Serum levels of VEGF-C in patients and healthy controls

The mean levels of serum VEGF-C for the different groups are reported in Table II. No statistically significant difference in VEGF-C levels was found between SpA, SC or CD patients compared with healthy subjects, although VEGF-C levels were higher in SC patients (Table II).

Relationship between circulating LEPC and VEGF-C levels and clinical parameters

We analysed circulating LEPC and VEGF-C levels in the three different groups of patients on the basis of different clinical parameters.

Concerning SpA clinical activity, the levels of LEPCs and VEGF-C did not correlate with BASDAI both in SpA and SC patients. According to BASDAI, SpA and SC were also analysed together: 27 patients were <4 (48.2%) and 29 >4 (51.8%). LEPC levels were higher in SpA and SC in comparison to controls, both in active (p=0.0025) and inactive (p=0.0070) disease, without differences between patients with BASDAI <4 and >4. No significant

Table II. LEPC and VEGF-C levels in the different groups of subjects. Data are represented as mean ± SEM, 95% CI and range. LEPC counts are reported as a percentage of total mononuclear cells, and VEGF-C levels in pg/ml.

	Mean	SEM	Lower 95% CI	Upper 95% CI	Minimum	Maximum
LEPCs						
Controls	2.00	0.4237	1.113	2.887	0	5.90
SpA	9.242**	1.878	5.428	13.06	0	43.10
SC	5.422*	1.038	3.232	7.613	0.70	17.20
CD	3.048	0.7987	1.391	4.704	0	11.70
VEGF-C						
Controls	1026	140	708.9	1342	487.5	1740
SpA	993.4	86.40	817.8	1169	115.5	2082
SC	1447	357.7	660	2235	512.8	5114
CD	1270	250.9	742.4	1797	124.4	3962

CD: Crohn's disease; LEPCs: lymphatic endothelial progenitor cells; SpA: spondyloarthritis; SC: spondyloarthritis associated with CD; VEGF-C: vascular endothelial growth factor-C; *$p<0.05$, **$p<0.001$ *vs*. controls.

difference in both LEPC and VEGF-C levels was noted between isolated axial SpA and SpA associated with entheseal and peripheral involvement.

In CD and SC, according to CDAI, disease activity was remittent in 25% of patients (n=12), mild in 22.9% (n=11), moderate in 14.6% (n=7), and severe in 37.5% (n=18).

For both LEPCs and VEGF-C, no difference between healthy controls and patients with different bowel disease's activity was noted, and no difference was found either comparing patients in remission or patients with low/moderate/severe activity, respectively.

For both LEPCs and VEGF-C, no significant difference between early (<10 years) and late (>10 years) disease was found in all patient groups. Disease duration did not correlate with LEPC and VEGF-C levels, except for CD patients, in which an inverse correlation with LEPC counts was observed (Pearson r=-0.4668, p=0.0397). In fact, lower LEPC levels were found in longstanding disease patients (4.44±1.47 <20 years *vs*. 0.27±0.19 >20 years, p=0.018). However, in both groups (<and >20 years of CD disease duration), no significant difference was observed compared with healthy subjects.

Discussion

In our study, circulating levels of lymphatic endothelial precursors were significantly increased in patients with spondylitis involvement, both SpA and SC, while no significant difference was observed between CD patients and healthy controls. Conversely, VEGF-C levels were not significantly different between groups. Furthermore, we found no correlation for both LEPC counts and VEGF-C levels with disease activity and duration in all patient groups, except for longstanding CD that showed lower LEPC counts.

The insufficiency of functional lymphatic vessels is an important aspect of SpA and CD, leading to a failure of fluid uptake from inflamed tissues. SpA is characterised by enthesitis and synovitis, in which both blood and lymphatic vessels are involved (23). The pathological changes of inflamed entheses include thickening, oedema and increased vascularity of tendon insertions, as well reported in the literature by imaging and histological data (23). Previous studies supported the role of angiogenesis in the pathophysiology and activity of SpA. In fact, angiogenesis may contribute to the genesis of synovitis and enthesitis in SpA, due to its involvement in synovial membrane hypervascularity and excessive bone formation. However, endothelial progenitor cells were found similar to controls (24), while the angiogenic factor VEGF was elevated in both axial and peripheral SpA patients and correlated with disease activity (25), even if discordant results for VEGF levels have been also reported (26). Instead, lymphangiogenesis has been overlooked, except for one study on SpA synovial biopsies reporting an increased number of lymphatic vessels, with a positive correlation with the severity of synovial inflammation (17).

Acute and chronic inflammation might provoke the production of growth factors for lymphatic vessels, such as VEGF-C, leading to intense de novo lymphangiogenesis. In particular, lymphatic neovascularisation is relevant in different forms of chronic arthritis (27-29), including SpA with peripheral involvement (17). Furthermore, the subcutaneous oedema is one of the most distinctive clinical features in SpA. Indeed, it was hypothesised that subcutaneous tissue involvement in dactylitis might precede tenosynovitis and synovitis in early disease (18).

Interestingly, in our work, the increase in LEPC counts observed in SpA and SC patients might indicate an active lymphovasculogenic process (de novo generation of lymphatic vessels through circulating progenitor cells) which is important for the development of new lymphatic vessels, interdependent from lymphangiogenesis derived from pre-existing lymphatic vasculature (15).

Because lymphatic vessels play central roles in gastrointestinal function, lymphangiogenesis was investigated and supposed to be prominent in CD but its role is not yet well elucidated (19, 30). In active CD, an exuberant expansion of lymphatic vessels in ileal and colonic biopsies (31) was found, as well as an increase in serum VEGF-C levels in active disease (32). However, if the involvement of lymphatic vessels in CD represents a protective or pathologic aspect of the disease is still debated (33). Our results showed that circulating levels of LEPCs and VEGF-C were similar in CD and healthy subjects and did not show any correlation with gut disease activity. Interestingly, LEPC levels in CD were lower in longstanding disease (>20 years), but whether the role of lymphovasculogenesis/lymphangionenesis is relevant in earlier phase is difficult to interpret from our results. In fact, in both groups (< and >20 years of CD disease duration) no significant difference was found in comparison to healthy subjects. In SC

and CD patients, scored by CDAI, either LEPCs or VEGF-C were also not influenced by remittent and not remittent stages, while SpA and SC, scored by BASDAI, showed elevated levels of LEPCs in both active and inactive spondylitis.

Collectively, our data suggest an active mobilisation of lymphatic endothelial precursors in SpA and SC, even if not associated to a VEGF-C increase. Interestingly, elevated LEPC levels in SC are likely attributable to spondylitis rather than gut involvement, since no significant difference in LEPC levels was found between CD and healthy controls. However, concerning VEGF-C levels, we should consider that the ELISA assay used in this study measured total circulating VEGF-C and could not distinguish between mature and immature forms of this growth factor. Therefore, we cannot exclude possible differences among our study groups in the levels of the mature VEGF-C form, which is the one specifically involved in the induction of lymphangiogenesis.

In conclusion, close relationships between lymphovasculogenesis/lymphangiogenesis and inflammatory diseases might be postulated only in spondylitis, either in SpA or in SC, independently from disease duration and activity. Furthermore, LEPC counts and VEGF-C levels are not increased in CD and appear to be independent from gut disease activity, but their role should be deeper studied in the future on a larger number of patients to better understand their possible involvement, overall in early disease.

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