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Endothelial progenitor cells and vascular endothelial growth factor in patients with Takayasu's arteritis

Luiz Samuel Gomes Machado*, Ana Cecilia Diniz Oliveira, Patricia Semedo-Kuriki, Alexandre Wagner Silva de Souza and Emilia Inoue Sato

Abstract

Background: Endothelial progenitor cells (EPCs) are responsible for endothelial damage repair. Takayasu's arteritis (TA) is a chronic inflammatory disease that affects large vessels. The aim of the study was to evaluate the number of EPCs and the levels of vascular endothelial growth factor (VEGF) and the relationship of these variables in patients with TA.

Methods: Thirty women with TA and 30 healthy controls were included. EPCs were assessed by flow cytometry and cell culture and VEGF quantification was performed by commercial ELISA kits.

Results: Ages of patients and controls were similar. The number of EPCs in patients and controls (median (interquartile range) were 0.0073% (0.0081%) vs. 0.0062% (0.0089%), $p = 0.779$ by flow cytometry and 27.0 (42.3) colony forming units (CFUs) vs. 27.0 (20.5) CFUs, $p = 0.473$ by cells culture, respectively. VEGF levels in patients and controls was 274.5 (395.5) pg/ml vs. 243.5 (255.3) pg/ml, $p = 0.460$. There was no difference in the number of EPCs and VEGF level between patients with active and inactive disease. There was a tendency of the number of angioblast-like EPCs in patients taking anti-TNFs to be higher; and in patients using methotrexate to be lower.

Conclusion: No significant difference was found in the quantification of EPCs and VEGF levels in TA patients compared to controls, and no difference was observed between patients with active and inactive disease.

Keywords: Endothelial cells, Vascular endothelial growth factor, Takayasu's arteritis

Background

Takayasu's arteritis (TA) is a chronic granulomatous vasculitis that affects mainly large vessels as aorta and its branches. TA patients also present premature atherosclerosis [1–4], as reported in other inflammatory rheumatic diseases [5, 6]. The pathogenesis of atherosclerosis in this arteritis is likely multifactorial and may be related to inflammatory process of vessels, chronic systemic inflammation and increased traditional cardiovascular risk factors [3, 7, 8]. In some diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), premature atherosclerosis is explained in part by decreased levels of endothelial progenitor cells (EPCs) [9–11].

The EPCs are bone marrow-derived cells that contribute to the reendothelialization of injured vessels, as well as for neovascularization after ischemic injury [12]. These cells are rare [13] and are considered independent predictors of morbidity and mortality in patients with cardiovascular disease [14]. There are two main types of EPCs: angioblast-like EPCs, as assessed by flow cytometry and monocytic EPCs as measured by colony forming units (CFUs) in cell culture [13]. VEGF and stromal cell-derived factor-1 (SDF-1) are hypoxia-induced oxygen-sensitive cytokines [15] and play a key role in the mobilization of EPCs from the bone marrow, maturing these into mature endothelial cells and targeting to sites with ischemic tissue [16, 17].

Studies have shown traditional cardiovascular risk factors reduce the count of EPCs [18–21] while medication as statins [22] and angiotensin converting enzyme inhibitors [23],

* Correspondence: luizmachado67@gmail.com
Rheumatology Division, Escola Paulista de Medicina UNIFESP (Universidade Federal de São Paulo), Rua Doutor Diogo de Faria, 561, apt 12, Vila, Clementin São Paulo-SP CEP: 04037-000, Brazil

neoplastic diseases [24, 25] and aerobic activity [26] increase the count of EPCs.

In relation to rheumatic diseases, most studies have shown a smaller number of EPCs in patients than controls in RA [9, 27, 28], SLE [10, 11, 29–31], thromboangiitis obliterans [32], ANCA-associated vasculitis [33, 34], Kawasaki disease [35] and Behçet's disease [36]. On the contrary, most studies in patients with systemic sclerosis (SSc) showed a greater number of EPCs when compared to controls [37–42].

In 2014, Dogan et al. published the first study evaluating EPCs in TA patients in Turkey and found no significant difference between TA and controls. However, the number of EPCs measured by flow cytometry in patients with active disease was higher than healthy control. Similar results were found for VEGF level [43].

In analogy to other inflammatory rheumatic diseases, we hypothesized that EPCs could also be involved in the physio pathogenesis of TA. The aim of the study was to quantify both angioblast-like and monocytic EPCs and VEGF levels in Brazilian patients with TA, as well to assess the number of EPCs and VEGF levels in relation to disease activity, presence of hypertension and dyslipidemia, and the use of medications.

Methods

This was a cross-sectional study carried out at the Federal University of São Paulo/Hospital São Paulo. All participants signed the consent form approved by the Ethics Committee of the institution. Participants were 30 women with TA, aged between 18 and 50 years, who met the classification criteria for TA of the American College of Rheumatology [44]. The control group consisted of 30 female healthy volunteers matched for age. We excluded patients who were current smokers, individuals with diabetes, end-stage kidney disease, coronary disease, infection, malignancy, with another autoimmune rheumatic disease or who had used cyclophosphamide until three months before the study.

TA activity was assessed by criteria of the National Institute of Health (Kerr' criteria) 1994 [45]. Thirty-five mL of peripheral blood was collected from all participants for cells count, erythrocyte sedimentation rate (ESR), creatinine, glucose and serum cholesterol and triglycerides, as well as to quantify angioblast-like and monocytic EPCs and to measure VEGF levels.

Quantification of angioblast-like EPCs by flow cytometry

Angioblast-like EPCs was quantified from peripheral blood mononuclear cells (PBMCs) isolated through density gradient medium with Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and stored at − 80 °C for 2 to 6 weeks. After thawing, the cells were incubated with 7AAD (Southern Biotechnology Associates Inc., Alabama, USA), anti-KDR-APC (R & D Systems, Inc., Minnesota, USA), anti-CD34-FITC (Southern Biotechnology Associates Inc.) and anti-CD133-PE (Miltenyi Biotec, California, USA) for 40 min. The quantification of EPCs was performed using a FACS Canto II cytometer (BD Becton Dickinson, California, USA). EPCs were defined as 7AAD-negative, CD34-positive, CD133-positive and KDR-positive lymphmononuclear cells [13]. The percentage of viable cells (7AAD-negative) were similar between patients and controls (50.1 (11.4)% vs 51,0 (14.1)%, respectively; $p = 0.684$).

Analyzes were performed using the Flow-Jo software program (Oregon, USA) acquiring on average, 450,000 events for each sample. The technique of fluorescence minus one (FMO) was used for the final analysis. The EPCs quantification was presented as a percentage of the absolute number of EPCs among viable lymphomononuclear cells.

Quantification of monocytic EPCs by cell culture

Monocytic EPCs was quantified according the literature [13]. PBMCs were incubated in 6-well plates coated with fibronectin (BD - Becton Dickinson) for 48 h at 37 °C. Each well contained 5×10^6 PBMCs suspended in 2 mL of Endocult medium (Stemcell Technologies, Washington, USA) supplemented with penicillin G streptomycin + amphotericin B (Invitrogen, California, USA). After incubation, the supernatant was aspirated, and a new cell count was performed and 1×10^6 cells were added in fibronectin-coated plates with 24 wells (BD - Becton Dickinson) suspended again in endothelial cell medium with antibiotic. Incubation was performed for another 72 h and stained with Giemsa 1% (EMD Chemicals Inc., New Jersey, USA). CFU counts were carried out though of inverted microscope (CK2, Olympus, New York, USA). CFUs were characterized by cell clusters surrounded by elongated and spiculated cells and the results are presenting as number of CFUs.

Confirmation of the endothelial lineage of CFUs

To confirm the endothelial lineage of CFUs, the same procedure for EPCs culture was conducted, with two incubations performed in 48 and 72 h, but in the last incubation fibronectin-coated plates with 24 wells were replaced by glass slides coated with fibronectin (BD BioCoat Fibronectin Coated Coverslips). After 72 hs incubation, coverslips were incubated with 12 μg/mL of 1,1′–dioctadecyl-3,3,3′,3-tetramethyl-indocarbocyanine perchlorate labeled acetylated low-density lipoprotein (Dil-Ac-LDL) (Invitrogen) for 4 h, fixed with methanol and incubated again with 10 μg/mL fluorescein isothiocyanate-conjugated *Ulex europaeus* agglutinin type I (FITC-UEA-I) (Sigma, Missouri, USA) [46]. Both are markers of endothelial lineage cells. Observation

and image capture was performed by immunofluorescence-specific microscopy (Carl Zeiss, Oberkochen, Germany) and confirmed that the cells which constituted the UFCs were endothelial lineage cells.

Quantification of VEGF

VEGF dosage was performed by ELISA using a commercial kit (Human, VEGF Quantikine ELISA - R & D Systems) according to manufacturer's manual.

Statistical analysis

Statistical Package for the Sciences (SPSS) version 15.0 (Chicago, USA) was used for statistical analysis. All data were considered having non-normal distribution by Kolmogorov and Shapiro-Wilk tests. Then, data were shown as median and interquartile range. Mann-Whitney *U* test was used for comparisons regarding the quantification of EPCs and VEGF levels between groups of patients and controls as well as among subgroups of patients. Chi-square and Fisher's exact test were used for comparisons of categorical variables between subgroups of patients. Values of $P < 0.05$ were considered significant and values between 0.05 and 0.10 were considered as a trend toward significance.

Results

The mean age of the patients and controls were comparable (32.5 (15.3) vs. 30.0 (5.3) years; $p = 0.646$). The mean time of diagnosis was 8.3 ± 6.5 years. Twenty-three (76,7%) patients were hypertensive [47], 18 (60%) were dyslipidemic [48] and 8 (27%) were obese (47). As expected the frequency of these variables were higher than in controls (Table 1).

With respect to medication 16 patients were using statins (53.3%), all were using acetyl salicylic acid (100%) and 22 were on some antihypertensive medication (73.3%). In relation to the specific treatment of arteritis, 23 patients (76.7%) were using corticosteroids and 24 were using immunosuppressive drugs and five were using anti-TNF (Table 2). According to Kerr's criteria, nine patients were classified as having active disease (30%).

No significant differences were found when comparing the subgroups of patients with and without disease activity in relation to hypertension, dyslipidemia, and use of statin, antihypertensive drugs, different doses of corticosteroids and different immunosuppressive drugs (Table 2).

EPC count and VEGF dosage in patients and controls

Flow cytometry showed that the proportion of EPCs between lymphomononuclear-viable cells was 0.0073% (0.0081%) in patients and 0.0062% (0.0089%) in controls ($p = 0.779$). In cell culture the mean of colony-forming units of EPCs were 27.0 (42.3) in patients and 27.0 (20.5) CFUs in controls ($P = 0.473$).

There was no difference in VEGF levels between patients and controls **(274.5 (395.5) pg/ml vs. 243.5 (255.3) pg/ml, $p = 0.460$).**

EPCs and VEGF in patients with and without active disease

Quantification of EPCs in cell culture and by flow cytometry, as well as VEGF levels did not differ between patients with and without disease activity (Table 3).

Medications

Comparing the subgroup of patients with and without anti-TNFα, no difference was found in relation to the quantification of monocytic EPCs and VEGF dosage. However, there was a tendency for patients using anti-TNF-α to have a higher number of angioblast-like EPCs (Table 4).

The use of methotrexate did not affect the quantification of EPCs by cell culture or VEGF dosage. However, we observed a tendency of patients using this medication to have a lower number of EPCs assessed by flow cytometry (Table 5).

There was no difference in the number of angioblast-like and monocytic EPCs when comparing subgroups of patients with and without use of leflunomide, statins and different doses of prednisone (data not shown).

Table 1 Demographic and clinical characteristics of the Takayasu's arteritis patients and controls

Variables	Takayasu's arteritis patients (30)	Controls (30)	*p*
mean age (years)	(32.5 (15.3)	30.0 (5.3)	0.646
mean time of diagnosis (years)	8.3 ± 6.5	_	
Skin color	White:18 (60%) Not White:12 (40%)	White: 20 (66%) Not White: 10 (34%)	0,592
patients with disease activity	9	_	
obesity (BMI > 30)	8 (27%)	1 (3%)	0,01
Hypertension	23 (76%)	2 (6%)	0,001
dyslipidemia	18 (60%)	4 (13%)	0,001

BMI Body Mass Index

Table 2 Frequency of hypertension, dyslipidemia, and use of medication in patients with and without Takayasu's arteritis activity

Variables	Without activity *N* = 21 *N* (%)	With activity *N* = 9 *N* (%)	*p*
Hypertension	16 (76.2)	07 (77.8)	1.00
Dyslipidemia	12 (57.1)	06 (66.7)	0.704
Statins	11 (52.4)	05 (55.6)	1.00
ACE inhibitors	10 (47.6)	05 (55.6)	1.00
ARB	02 (9.5)	01 (11.1)	1.00
Beta-blockers	05 (23.8)	04 (44.4)	0.389
Calcium channel blockers	05 (23.8)	03 (33.3)	0.666
Acetyl salicylic acid	21 (100)	09 (100)	1.00
Prednisone	16 (76.2)	07 (77.8)	0.166
	Dose ≤5 mg: 06 (37.5)	Dose ≤5 mg: 0	
	Dose > 5 mg: 10 (62.5)	Dose > 5 mg: 07 (100)	
Immunosuppressive drugs	17 (80,9)	07 (77,7)	1,00
	Leflunomide: 06 (35)	Leflunomide: 05 (71,4)	0,225
	Methotrexate: 09 (53)	Methotrexate: 01(14,2)	0,204
	Azathioprine: 02 (12)	Azathioprine: 01 (14,2)	1,00
users of anti-TNF	03 (14,2)	02 (22,2)	0,622

ACE angiotensin-converting enzyme, *ARB* angiotensin receptor blocker
TNF tumor necrosis factor

Hypertension and dyslipidemia

There were no differences in the number of angioblast-like and monocytic EPCs, as well as the levels of VEGF when compared TA patients with and without hypertension and dyslipidemia (data not shown).

Discussion

Contrary to our expectation, and in opposition of the only one study in the literature [43], in the present study TA patients showed no significant difference compared to healthy women with respect to quantification of EPCs by flow cytometry, which evaluates angioblast-like EPCs or by cells culture with early growth, which evaluates monocytic EPCs. There was also no significant difference in relation to VEGF dosage between groups.

TA is a chronic inflammatory disease associated with elevated levels of several inflammatory cytokines [49, 50]. In our previous study TA patients with active disease showed increased TNF and IL-6 levels compared to inactive [51]. Many studies have evaluated EPCs in other rheumatic diseases. Holmen et al. (33) showed than the numbers of EPC colony-forming units are lower in patients with active granulomatosis with polyangiitis (GPA) as compared with those in remission and healthy individuals, probably caused by high levels of IL-8, epithelial neutrophil activating peptide-78 (ENA-78), macrophage inflammatory protein-1α (MIP-1α), and growth-related oncogene-α (GRO-α) found in supernatants from patients with active disease. Patients with RA also have a lower number of EPCs than healthy individuals [9, 27, 28], however in RA, different than SLE, the cytokine responsible for the reduction of these cells seems to be TNF-α [52, 53]. Since TA is

Table 3 EPCs and VEGF in Takayasu's arteritis patients with and without disease activity

Variables	With activity *N* = 09 Median (IQ)	Without activity *N* = 21 Median (IQ)	*p*
Monocytic EPCs (number of CFUs)	27.0 (40.0)	27.0 (43.0)	0.929
Angioblast-like EPCs (%)	0.0038 (0.0067)	0.0074 (0.0118)	0.326
VEGF (pg/ml)	279.0 (625.0)	272.0 (295.0)	0.657

IQ Interquartil Range
EPCs endothelial progenitor cells
VEGF vascular endothelial growth factor

Table 4 EPCs and VEGF in Takayasu's arteritis patients users and nonusers of anti-TNF

Variables	With anti-TNF *N* = 5 Median (IQ)	Without anti-TNF *N* = 25 Median (IQ)	*p*
Monocytic EPCs (number of CFUs)	49.0 (59.5)	27.0 (39.0)	0.706
Angioblast-like EPCs (%)	0.0108 (0.0068)	0.0038 (0.0079)	0.085
VEGF (pg/ml)	272.0 (478.0)	277.0 (453.0)	0.787

IQ Interquartil Range
EPCs endothelial progenitor cells
VEGF vascular endothelial growth factor

Table 5 EPCs and VEGF in Takayasu's arteritis patients users and nonusers of methotrexate

VARIABLES	With methotrexate $N = 10$ Median (IQ)	Without methotrexate $N = 20$ Median (IQ)	*p*
Monocytic EPCs - (number of CFUs)	27.5 (34.3)	27.0 (43.2)	0.746
Angioblast-like EPCs - (%)	0.0033 (0.0065)	0.0088 (0.0098)	0.061
VEGF (pg/ml)	217.5 (419.8)	293.0 (442.3)	0.286

IQ Interquartil Range
EPCs endothelial progenitor cells
VEGF vascular endothelial growth factor

a chronic inflammatory disease, where increased levels of TNF-α and other cytokines are also described [49, 50], we expected to find lower numbers of EPCs in this vasculitis.

The absence of the difference in the quantification of EPCs between TA patients and healthy controls can be explained, in part, by the balancing between intrinsic factors of Takayasu's arteritis, some of them that inhibit the formation of EPCs and others that stimulate their formation. Among the inhibiting factors, the most relevant are the chronic inflammatory nature of this disease [49, 50] and the high prevalence of hypertension and dyslipidemia. Regarding stimulatory factors, the most important is the chronic ischemic state observed in this arteritis [45]. Chronic ischemic states, such as those that occur in systemic sclerosis (SSc), promote the increase of EPCs [37–42]. Del Papa et al. [40, 41] reported a significant increase in VEGF, a cytokine-induced hypoxia, in SSc patients [15], which was associated with elevated levels of EPCs, thus showing the importance of hypoxia as a stimulator of EPCs. Although SSc and TA affect vessels with different caliber, ischemia/chronic hypoxia also can occur in TA, which could, in theory, contribute to the increase in EPCs. Usually TA patients with severe arterial stenosis/occlusion present collateral arteries formation that needs the presence of VEGF and EPCs.

The concomitant presence of inflammation and ischemia in TA may also explain the lack of difference in the levels of VEGF compared to controls. Inflammation, beyond reducing the number of EPCs, may be at least partially responsible by the reduction in VEGF serum levels, as observed in SLE patients [29]. Ischemia, in turn, can raise levels of this angiogenic cytokine, a situation found in systemic sclerosis [40, 41].

To assess the influence of inflammation on the quantification of EPCs, we analyzed subgroups of patients divided according to disease activity and no difference was observed between patients classified as active and inactive disease using Kerr's criteria. We know these criteria fail in the characterization of disease activity [44], but there is no criterion considered ideal. Therefore, the formation of subgroups may not have been adequate, which may have affected the outcome.

The Dogan study [43] found no difference of EPC between TA patients and controls, however they found a higher EPC number measured by cytometry, in subgroup of active disease comparing to controls. They also found higher levels of VEGF in TA patients with active disease than controls. This study presented a different patient's profile. Their patients were older, and smoker, diabetes and coronary disease were not excluded, for other hand our patients had higher frequency of dyslipidemia and hypertension. However, all these conditions are associated with lower EPC number and cannot explain the difference between studies. Although sample size and the methods to EPC measuring were similar, some difference between our and their study could be due to the ethnic variability. They did not evaluated the monocytic EPC by culture, than we could not compare with our result.

As several TA patients had factors known to influence the quantification of EPCs, we made comparative analysis between patients with and without hypertension and dyslipidemia, and no difference was observed between these subgroups. This results are according with Dogan study, which also did not find differences between patients with and without cardiovascular risk factor. One possible explanation for these results is that the majority of hypertensive and dyslipidemic patients were using medications, such as statins and ACE inhibitors, and studies have shown that the use of these medications increases quantification of EPCs [22, 23].

There was a tendency of increased angioblast-like EPCs in the subgroup of TA patients using anti-TNFα therapy, suggesting the importance of inhibition of this cytokine to increase EPCs. In RA patients, Ablin et al. [53] reported increased number of EPCs, assessed by cell culture, after infliximab use. Furthermore, Grisar et al. [52] demonstrated in vitro that cultured EPCs in the presence of TNFα showed reduced formation of CFUs.

The subgroup of TA patients using methotrexate showed a trend to have fewer angioblast-like EPCs when compared to patients without this medication. Only one study assessed the effect of methotrexate in cultured EPCs, observing an increase in apoptosis of those cells. [27]. This may be a possible mechanism to explain our data.

The limitations of our study were: a) a small sample due to the rarity of the disease; b) the use of frozen cells for cell counts, reducing the number of lymphomononuclear-viable cells, however, there was no difference in the percentage of viable cells between patients and controls, and; c) the cross-sectional design to assess disease activity and effect of medication. Ideally, a prospective study would better evaluate the effect of these variables.

Conclusion

In conclusion, no significant difference was found in the quantification of EPCs and VEGF levels in TA patients compared to controls, and no difference was observed between patients with active and inactive disease.

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Authors' contributions

LSGM did a literature review, wrote the article and participated in flow cytometry and cell culture; ACDO did the clinical evaluation of patients; PSK developed the standardization of flow cytometry and cell culture; AWSS support in statistical analysis and bibliographic review; EIS idealized the project and did revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Federal University of São Paulo/Hospital São Paulo - CEP 1285/09.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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