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Increased levels of circulating endothelial cells in chronic periaortitis as a marker of active disease

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Background. The pathogenesis of chronic periaortitis (CP) has not been clarified. The histologic features and the association with autoimmune diseases suggest an immune-mediated disorder with marked inflammatory vascular and perivascular lesions. To clarify the role of vascular damage we looked for the presence and the surface phenotype of circulating endothelial cells (CECs) in the peripheral blood of patients with chronic periaortitis.

Methods. Eleven patients with CP were evaluated for the presence of CECs; 9 patients had active and 2 inactive disease. Three patients with active disease were also evaluated 3 months after therapy. Ten atherosclerotic patients, 10 patients with renal insufficiency of variable degree and etiology, and 40 healthy subjects were evaluated as controls. Five-parameter, 3-color flow cytometry was performed with a FACScan. CECs were defined as CD45 negative, CD31, P1H12, and CD36 positive, and activated CECs as CD45 negative and P1H12, CD62 positive.

Results. The median number of CECs in patients with CP (10^6 cells/mL) was significantly higher than in healthy controls (16 cells/mL, $P = 0.0004$) and atherosclerotic patients (25 cells/mL, $P = 0.0005$) Two patients with inactive disease had a CEC count comparable to that of normal subjects. In 2 of the 3 patients reevaluated, 3 months after therapy CEC numbers normalized. Almost all CECs were microvascular in origin and showed an activated phenotype.

Conclusion. The presence of a high number of CECs in the active phase of chronic periaortitis and their normalization during inactive disease suggest that endothelial damage may play a role in the pathogenesis of the disease.

The term chronic periaortitis (CP) refers to a family of inflammatory diseases with similar morphologic appearance, namely idiopathic retroperitoneal fibrosis, perianeurysmal retroperitoneal fibrosis, and inflammatory aneurysm of the aorta [1]. The clinical presentation may be different according to the anatomic location. When the ureter is involved, pain in the low back region is common, and may be associated with low-grade fever and weight loss. An obstructive uropathy usually develops, which may remain asymptomatic until it leads to renal insufficiency. Arterial invasion with aortic aneurysm, or portal hypertension and/or bile duct obstruction with pseudotumor of pancreas, as well as extrinsic venous compression with deep venous thrombophlebitis of legs, may also occur. Ultrasonography may show hydronephrosis and intravenous urography may show a medial deviation of the ureter in the case of retroperitoneal fibrosis. However, the identification of the retroperitoneal mass is made by computed tomography or magnetic resonance. Once the mass has been identified the main problem is the differential diagnosis between CP and a retroperitoneal tumor. Laparatomy and multiple deep biopsies are required to ascertain the diagnosis. Surgical treatment with blunt dissection can be successful, but in many cases fibrosis recurs months or years later. Steroid therapy is often used either as primary therapy or in adjunct to surgery. Immunosuppressive agents and tamoxifen may also be helpful [2].

The diseases classified as CP have in common the histopathologic characteristics of severe adventitial and perivascular inflammatory infiltrate, which evolves throughout fibrosis around atherosclerotic vessels. Because of these histologic features, CP has been classified among the scleroderma like disorders [3]. The pathogenesis of CP remains unknown. However, the reported association of CP with vasculitic syndromes [4] or autoimmune diseases [5–7], and the positivity of antinuclear antibodies (ANA) and anti-neutrophil cytoplasmic antibodies (ANCA) in a few patients with CP [8–11], may

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suggest a role for immunologic mechanisms in the pathogenesis of the disease.

In the present study, we assessed by cytometric method the presence and the surface phenotype of circulating endothelial cells in patients with CP. We also evaluated whether the presence of CECs is associated with the active phase of the disease and can be used as a marker of the immune-mediated vessel damage.

METHODS

Patients and controls

Patients with a diagnosis of chronic periaortitis based on computed tomography (CT) were selected for this study. An informed consent was given by all patients. At the moment of investigation, none of the patients were taking any medication potentially responsible for chronic periaortitis. Infection and malignancy were excluded on clinical and laboratory grounds, and with a histologic evaluation in 7 out of 11 patients. Five histologic samples were obtained during surgery and 2 by laparoscopy. The following definitions were adopted: (*1*) "active disease," characterized by the presence of a periaortic mass englobing 1 or both ureters with hydronephrosis at CT scan plus an increase in C-reactive protein (CRP) and/or erythrocyte sedimentation rate (ESR); (*2*) "inactive disease," characterized by the regression of hydronephrosis, and by a marked reduction of fibrotic tissue at CT scan in comparison to the basal examination together with the normalization of CRP and ESR; (*3*) "reactivation of the disease," when after at least 1 year of quiescence a control CT scan showed a new increase of the periaortic mass englobing 1 or both ureters associated with a new increase in CRP and/or ESR.

The clinical and laboratory data collected for this study are those obtained at the time the blood samples were taken.

We used 3 groups of controls, patients with atherosclerosis, patients with renal insufficiency, and normal subjects. Ten patients (8 males and 2 females, median age of 64 years, range 54–78 years) with a history of atherosclerotic vascular disease were examined as vascular disease controls. Three out of 10 patients had atherosclerotic coronary artery disease, 4 had femoral atherosclerosis with claudication in 2, and 4 had carotid stenosis**.** A computed tomography was done on all these patients to exclude the presence of chronic periaortitis. Ten patients (7 males and 3 females, median age of 66 years, range 36–74 years) with renal insufficiency of variable degree (median plasma creatinine 4.5 mg/dL range 1.6-9 mg/dL) and etiology (chronic interstitial nephritis 3 patients, nephroangiosclerosis 3 patients, diabetic nephropathy 2 patients, polycystic kidney disease 2 patients) were used as controls with renal insufficiency. Forty sex- and age-matched healthy subjects were investigated as normal controls.

Autoantibody profile

ANA and anti-dsDNA antibodies were assessed by indirect immunofluorescence using Hep-2 cells and *Chritidia lucilliae*, respectively. Antiextractable nuclear antigen antibodies were measured by enzyme-linked immunoabsorbent assay (ELISA) (Diamedix, Miami, FL, USA). ANCA were detected by indirect immunofluorescence on fixed neutrophils and by ELISA using proteinase 3 and myeloperoxidase as antigens [12].

To assess whether sera from CP patients displayed the same reactivity against endothelial cells (EC) obtained from different human anatomic sources, we evaluated the AECA activity of CP sera against large arterial (aorta) and microvascular EC. AECA were detected using a cell surface ELISA on macrovascular (human aortic endothelial cells) and microvascular (human dermal-derived endothelial cells, d-HMEC) confluent living endothelial cells, as previously described [13]. All the above-described parameters were measured in each patient.

Detection of CECs by flow-cytometry analysis

Circulating endothelial cells were detected directly on whole peripheral blood by flow cytometry, as previously described [14]. Briefly, 200 μL of peripheral blood samples in sodium heparin was labeled with 10 μL of a panel of fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), or peridin chlorophyll protein (PerCP)-conjugated antibodies anti-CD45, -CD31, -CD62, -CD36, and -P1H12 for 20 minutes at room temperature. After conjugation, red blood cells were lysed by incubating in FACS lysing solution (Becton Dickinson, San Jose, CA, USA) for 15 minutes at room temperature. White blood cell pellets were then washed twice in FACS flow solution (Becton Dickinson).

Appropriate analysis gates, designed to remove dead cells, platelet aggregates, and debris, and to exclude CD45+ hematopoietic cells, were used to enumerate total and activated CECs. CECs were defined as CD45-negative and CD31-, P1H12-, and CD36-positive [14]. Activated CECs were defined as P1H12- and CD62-positive. Five-parameter, 3-color flow cytometry was performed with a FACScan flow cytometer with a 15-mW argon laser (excitation at 488 mm) (Becton Dickinson). The sensitivity of fluorescence detectors was set and monitored using Calibrite Beads (Becton Dickinson) according to the manufacturer's recommendations. A vial with cells stained with IgG1 isotypic controls labeled with FITC or PE was used as negative control. At least 100,000 (usually 300,000) cells per sample were acquired, and analyses were considered informative when adequate numbers of events (>100) were collected in the CEC enumeration gates [13]. Data were analyzed with CellQuest software (Becton Dickinson).

Table 1. Clinical characteristics at time of CECs determination

PTS	Sex	AGE	Diagnosis	Ureteral involvement	Serum creat mg/dL	CRP	ESR	Auto-Abs	AVD
1[a]	F	60	IRPF	Left right	10	0.5	60	Anti-thyroid pos	
2[a]	F	51	IRPF	Left right	3.8		54	Anti-thyroid pos	Femoral
3[a]	M	65	IRPF	Left	0.9	120	86	ANA pos	
4[a]	M	69	IRPF	Left right	1.7	1.5	22	ANA pos	
5[a]	M	64	IRPF	Right	1.98	2	30	Anti-thyroid pos	Carotid
6[b]	M	72	IRPF	Right	1.4	1.4	62		Femoral
7[b]	M	65	IRPF	Right	1.6	7.7	70	AECA pos	
8[b]	M	63	Inflam A	Right	4	1.5	40	Anti-thyroid pos	
9[b]	M	48	IRPF	Left	1	7.6	15		Coronary
10[c]	F	58	IRPF	Left, right	1.4	0.1	7		
11[c]	M	76	IRPF	Left, right	3.2	0.2	12	AECA pos	
5[d]	M	64	IRPF	Right	1.1	0.2	11	Anti-thyroid pos	Carotid
6[d]	M	72	IRPF	Right	1.0	0.1	15		Femoral
7[d]	M	65	IRPF	Right	1.2	0.4	10		

Abbreviations are: M, male; F, female; IRPF, idiophatic retroperitoneal fibrosis; Inflam A, inflammatory aneurysm; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; Auto Ab, autoantibodies; Anti thyroid, antimicrosome and antithyreoglobulin antibodies; AECA, antiendothelial cells antibodies; AVD, atherosclerotic vascular disease.

[a]Patients evaluated at diagnosis of chronic periaortitis; [b]patients evaluated at reactivation of the disease; [c]patients with inactive disease; [d]patients reevaluated three months after therapy.

The concentration of CECs per mL blood was calculated according the following formula: $(\varepsilon/\nu) \times WBC \times 1000$, where ε is the number of CECs detected in the CD45-negative enumeration gate; ν is the total number of cells acquired per sample, and WBC is the number of circulating white blood cells per microliter.

Detection of soluble interleukin-6 (IL-6)

Plasma concentrations of IL-6 were assessed by ELISA (R&D Systems, Minneapolis, MN, USA), and calculated using a standard curve generated with specific standards.

Statistical analysis

Comparisons of CEC levels between CP patients, and patients with diffuse atherosclerosis and healthy controls were performed by the Mann-Whitney rank sum test. A P value < 0.05 was considered significant. In addition, correlations between CEC number and different CP disease parameters were assessed by Spearman's rank correlation test.

RESULTS

Demographic and clinical features of patients

The study cohort consisted of 9 men and 2 women, with a median age of 61 years (range 46–72 years). Ten patients had a diagnosis of idiopathic retroperitoneal fibrosis, and 1 patient had a diagnosis of inflammatory aneurysm of the aorta. The CT diagnosis rested on the demonstration of a periaortic soft tissue mass of variable thickness that enveloped the aorta and inferior vena cava between the renal hilar and sacral promontory and extended laterally to entrap the ureters, resulting in variable degrees of hydronephrosis in 10 cases, and associated with an aneurysm of the involved aorta in 1 case. At the time of diagnosis all patients had ureteral obstruction with unilateral hydronephrosis in 2 and bilateral hydronephrosis in the other 9. The latter 9 patients had impaired renal function (median plasma creatinine 2.2 mg/dL, 25th to 75th percentile 1.8-8.5 mg/dL). Three out of the 11 patients had deep vein thrombosis of the leg. Nine patients had an elevated ESR (median 40 mm/h; 25th to 75th percentile: 16.75-61.5), and 7 had an elevated CRP (median 6 mg/dL; 25th to 75th percentile: 2-29 mg/dL; normal value <0.5 mg/dL). Five patients were submitted to ureterolysis and transposition of the ureters with surgical repair of the abdominal aortic aneurysm in 1 of them. During surgery, multiple deep biopsies of the retroperitoneum were done. Two other patients were submitted to laparoscopy with multiple biopsies of the fibrotic mass. In the remaining 4 patients, the histologic evaluation of the fibrotic mass was not done. The CT did not show any sign of malignancy namely: a cranial location of the mass, anterior displacement of the aorta, lateral displacement of the ureters, and/or bone destruction [15]. These 4 patients were treated with steroids with marked reduction of the fibrotic mass and resolution of the hydronephrosis. They were followed in our unit from 24 to 43 months after the diagnosis of periaortic fibrosis without any sign of malignancy.

At the time of CEC determination, 9 patients had clear-cut evidence of active disease. Five of these 9 patients were studied at the moment of diagnosis of CP, and 4 during a reactivation of the disease 3.5 ± 3.5 years after the diagnosis (Table 1). The other 2 patients had inactive disease, respectively, 2 and 5 years after surgical and steroid treatment. Four patients had an associated

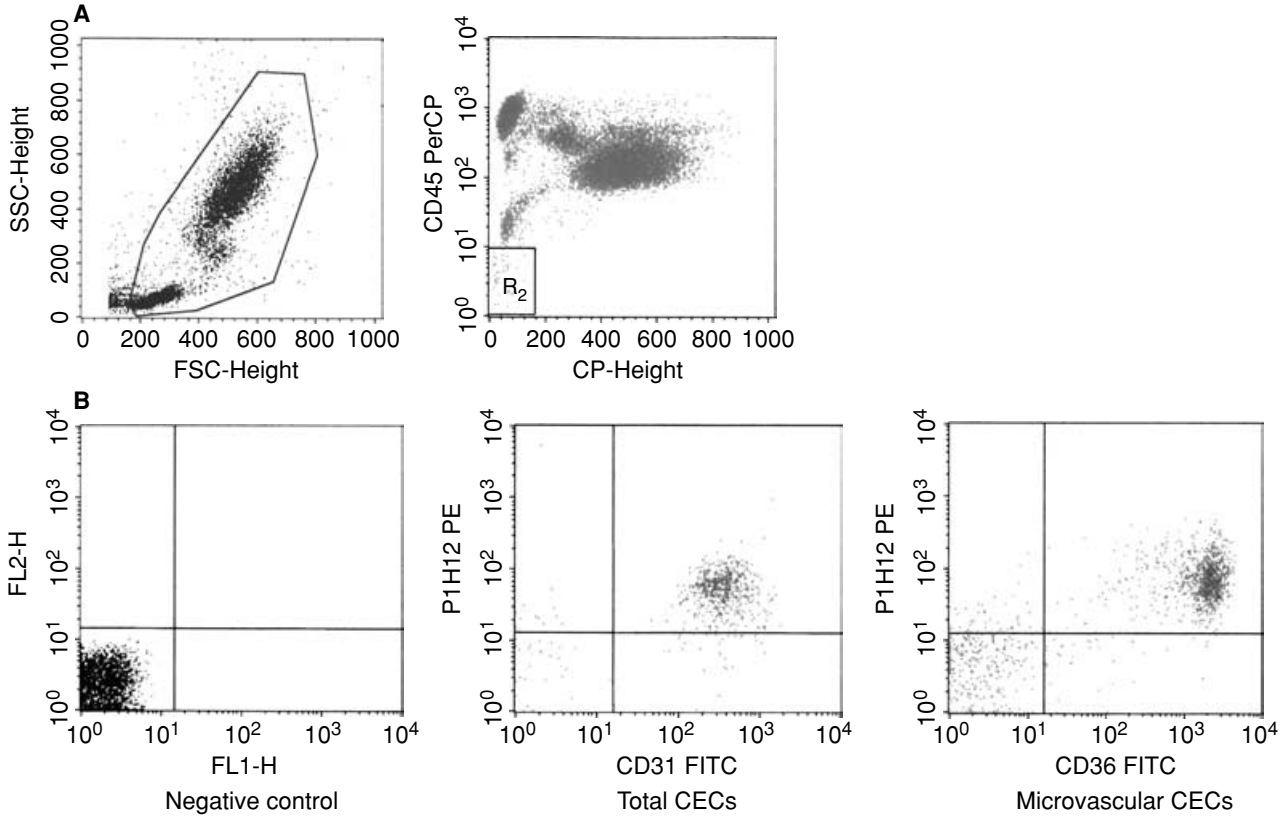

Fig. 1. Flow cytometry evaluation of circulating endothelial cells (CECs). (*A*) Representative panel showing the analysis gate used to exclude platelets and debris (left), and the gate used to exclude CD45-positive hematopoietic cells (right). (*B*) Representative panels showing the negative control, total, and microvascular CECs in chronic periaortitis. PerCP, peridin chlorophyll protein; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

atherosclerotic vascular disease, namely: femoral stenosis in 2 patients, carotid stenosis in 1 patient, coronary artery disease in 1 patient.

Three out of the 9 patients, first evaluated during active disease, had a second evaluation 3 months after beginning a treatment with steroids, and tamoxifen in 2 patients, and steroids and mycophenolate mofetil in the last patient.

Autoantibodies

Four patients had thyroid dysfunction with antithyroid microsome and antithyreoglobulin antibodies. As a consequence, 2 patients had hyperthyroidism and 2 had hypothyroidism. Two patients were ANA-positive with speckled pattern. Cryoglobulins, rheumatoid factors, ANCA, antibodies to ENA, and dsDNA were negative in all patients. Two patients were positive for AECA-IgG both on aorta and microvascular EC, at low titers.

CEC levels by flow-cytometry analysis

Using flow-cytometry analysis, we identified CECs as negative for hematopoietic marker CD45 and positive for endothelial markers P1H12 and CD34 (Fig. 1). The mean number of CECs in patients with CP (mean value 106/mL, 95% CI 70–143) was significantly higher than in healthy controls (16/mL, 95% CI 9–23; $P < 0.0004$), in patients with diffuse atherosclerosis (25/mL, 95% CI 16–33; $P = 0.0005$) as well as in patients with renal insufficiency (mean value 15/mL, 95% CI 8–29; $P = 0.005$). There was not any significant difference between healthy controls, patients with atherosclerosis, and patients with renal insufficiency. Interestingly, while the number of CECs was significantly higher than controls in patients with active disease (ranging from 90 to 242 cells/mL, median 108 cells/mL, mean 114 cells/mL, SD 46.2) (Fig. 2), the 2 patients in clinical remission had low CEC counts (31 and 37 cells/mL, respectively), comparable to those of atherosclerotic and normal subjects (Fig. 2). In 2 out of 3 patients with active disease, cell numbers declined after 3 months of treatment (from 108 to 34 cells/mL and from 105 to 6 cells/mL, respectively. No change in CECs levels was observed in the third patient before (90 cells/mL) and after therapy (85 cells/mL), in spite of normalization of C-reactive protein, erythrocyte sedimentation rate, and improvement of abdominal computed tomography (Fig. 3).

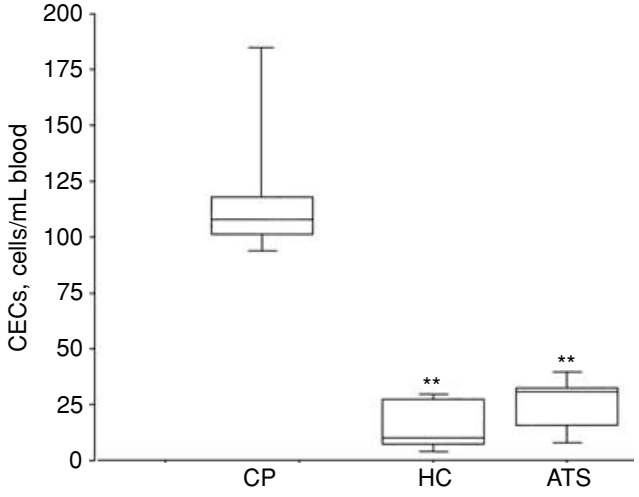

Fig. 2. Levels of circulating endothelial cells (CECs) in peripheral blood obtained from patients with active chronic periaortitis (CP), healthy (HC), and atherosclerotic (ATS) controls. Data are shown as box plots, with upper and lower quartiles (shaded areas) and medians (horizontal lines within shaded areas). The number of CECs in CP patients was significantly higher when compared with those in controls. $^{**}P < 0.001$.

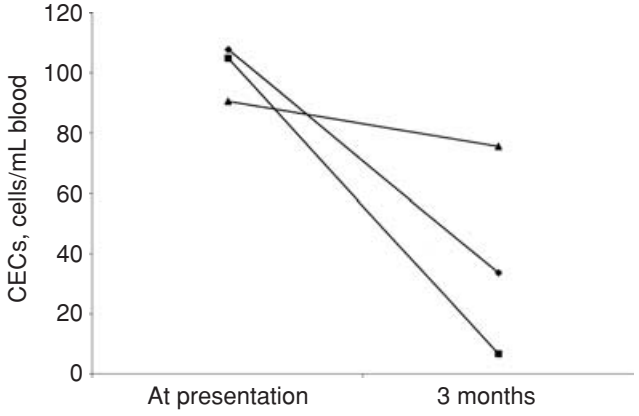

Fig. 3. Cell numbers during the course of immunosuppressive therapy in patients with active disease.

Phenotype of circulating endothelial cells

Among the various types of endothelial cells, only microvascular endothelial cells express CD36. We used this marker to identify the vascular origin of CECs in patients with CP. Almost all CECs of patients with CP expressed CD36. To evaluate the activated state, we analyzed CECs for dual expression of P1H12 and molecules that are expressed on the endothelial surface upon activation. The number of CECs with surface expression of E-selectin (CD62) had increased in patients with CP in comparison with healthy controls ($P = 0.005$) (Fig. 4). Activated CEC number and phenotype were higher in patients with an active disease than in patients in a steady state ($P = 0.005$, data not shown). No correlation between activated CECs counts and ESR or CRP was found.

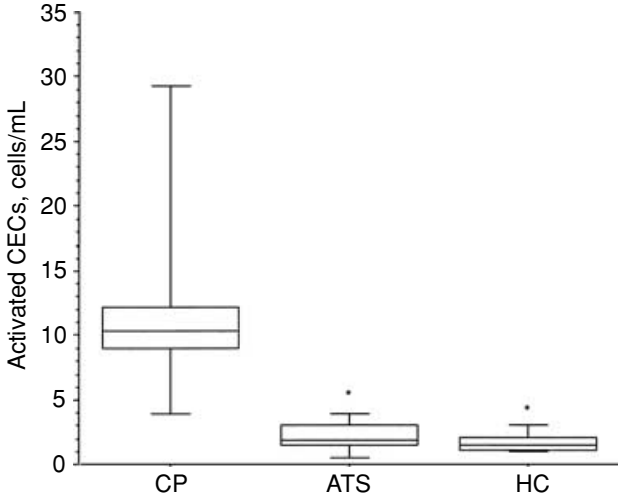

Fig. 4. The activated phenotype of CECs was defined by P1H12 and CD62 surface expression. Data are shown as box plots, with upper and lower quartiles (shaded areas) and medians (horizontal lines within shaded areas). The number of activated CECs was markedly increased in patients with CP in comparison with atherosclerotic (ATS) and healthy (HC) controls. $^{*}P = 0.005$.

Detection of plasma IL-6 levels

The mean (± SD) plasma concentration of IL-6 was 25.13 ± 20.1 pg/mL in CP patients, and 3.8 ± 3.7 in healthy subjects ($P < 0.0001$) and 4.1 ± 2.7 in vascular controls ($P < 0.0001$). No positive correlation was found between CECs and IL-6 levels.

DISCUSSION

The pathogenesis of CP remains unknown. The association of the disease with other autoimmune disorders [4–7, 16, 17], as well as the presence of serologic markers of immune activation in a small number of CP patients [8–11], suggests that CP could be an immune-mediated disorder with marked inflammatory vascular traits. The intrinsic mechanisms triggering the inflammatory processes in the adventitial and periadventitial vasa vasorum of the aorta are, at the moment, unknown. Parums et al [18] postulated that the disease may be due to an immune reaction to some components of atherosclerothic plaques (i.e., ceroids), which are a complex of proteins and oxidized LDL. This hypothesis has been partially confirmed by the finding of antibodies directed against ceroid in the sera of patients with CP. However, the same antibodies have also been detected in the sera of healthy elderly subjects and in patients with atherosclerosis [18, 19].

Recently, a significant number of CECs have been detected in different diseases, such as acute coronary syndrome, sickle cell anemia, rickettsia and cytomegalovirus infection, SLE, ANCA-associated small-vessel vasculitis, Kawasaki disease, and SSc [14, 10–27]. In addition, in a large group of patients with SLE, we found a number

of CECs higher than in normal controls [28], but lower with respect to that observed in patients with CP in the present study (mean values in SLE patients 44.5/mL, 95% CI 38.6–50.3). The cumulative significance of these studies supports the evidence that endothelial damage plays a key role in the pathogenesis of these diseases. Moreover, it is of interest that CEC numbers varied according to the severity of the endothelial lesions and was closely correlated with the degree of disease activity [14, 25–27].

It is worth noting that there are different methods used to measure CECs, none of which is standardized, so that it is difficult to compare one method to the others. The method we used is one of the most frequently used in recent studies. Although not validated, it allows reliable results in different disease groups to be obtained [29].

In this study, we found that patients with CP had a significantly increased number of CECs in comparison to healthy subjects, patients with diffuse atherosclerosis, and patients with renal insufficiency of variable degree and etiology. These data indicate that endothelial injury, as part of immune-mediated inflammatory vascular damage, may play a role in the pathogenesis of CP. Actually, there is growing evidence that CP belongs to a family of inflammatory vascular diseases characterized by marked adventitial and periadventitial infiltration by lymphocytes, plasma cells, and macrophages, involving abdominal aorta and retroperitoneal small vessels sometimes with clear vasculitic lesions [11, 30–32]. Due to these histologic features and the subsequent evolution of the inflammatory lesions to fibrosis, it has been proposed to classify CP among the scleroderma-like disorders [3]. As a matter of fact, SSc is a connective tissue disease characterized by an early phase of inflammatory microangiopathy followed by a large deposition of collagen fibrils and, finally, evolution to diffuse fibrosis [33, 34] involving skin and visceral organs. Instead, the localization of CP is limited to the collagen tissue around the abdominal aorta. Recently, CECs have been found in high number in SSc, and their amount was strongly correlated with active vascular disease and plasma markers of endothelial damage [14]. Our data would suggest that these 2 diseases share not only the histologic features, but also some pathogenetic mechanisms. As described in SSc, CECs in patients with CP seem to be a good marker of early microvascular damage, while their number decrease during the inactive phases of the disease or after an effective treatment. Although in our series the number of patients with active or quiescent disease was small, it is of interest that in 2 patients with inactive CP for more than 2 years the levels of CECs were low and comparable to controls. In the 3 patients tested before and 3 months after treatment, the level of CECs normalized in 2. These findings, together with the absence of a significant number of CECs in patients with diffuse atherosclerosis, which have been selected as vascular disease controls, seem to confirm that CECs can be considered as reliable markers of microvascular injury in CP.

As far as circulating endothelial phenotype is concerned, we found that almost all CECs in patients with CP are microvascular in origin, as defined by the marker CD36 [22, 35]. This finding could further suggest that vascular injury in CP takes its origin from vasa vasorum rather than the aortic layers. In addition, some CECs in patients with CP have an activated phenotype, as evidenced by the surface expression of E-selectin. Moreover, we found increased plasma concentrations of IL-6, which is an important cytokine, having a crucial role as an inflammatory mediator and modulating fibroblast properties [36, 37].

CONCLUSION

The present study shows that high counts of CECs are detectable in the peripheral blood of patients with CP, CECs are elevated in active phases of CP, and CEC levels normalized in inactive phases of CP and after immunosuppressive treatment. These data suggest that endothelial damage may play a key role in the pathogenesis of CP, and that the amount of CECs in the blood is strictly correlated with the activity of the disease. Further studies are necessary to evaluate whether CECs may be used to monitor the activity of CP.

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