High circulating levels of cytokines (IL-6 and TNFα), adhesion molecules (VCAM-1 and ICAM-1) and selectins in patients with peripheral arterial disease at rest and after a treadmill test

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Abstract: Peripheral arterial disease (PAD) is a common manifestation of atherosclerosis that is associated with systemic inflammation. The aim of our study was to assess whether plasma markers of inflammation increased after exercise in patients with PAD. The study was conducted on two groups of 20 subjects each: one group (mean age 68.4 ± 5.09 years) was affected by PAD with claudication, while the other group consisted of healthy controls (66.9 ± 6.1 years). Concentrations of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) were determined in plasma, in supernatants and in cells stimulated with 1 mg lipopolysaccharide in all patients. E-selectin (ES), L-selectin (LS) and P-selectin (PS) concentrations and plasma concentrations of VCAM-1 and ICAM-I were also determined. All determinations were performed in patients at rest and after the treadmill exercise. Resting values of soluble mediators were greater in PAD patients than in controls. They increased in both groups after the treadmill test, even if post-treadmill concentrations were significantly higher in PAD patients (PAD $p < 0.001$ or 0.0001, controls $p < 0.05$ or 0.001).

These results confirm that white blood cell activation is characteristic of systemic atherosclerosis and that these inflammation markers increase in conditions of hemodynamic stress.

Key words: inflammatory mediators; treadmill test; vascular disease

Introduction

Vascular diseases are characterized by the release of endothelial-derived adhesion cells and the presence of proinflammatory circulating molecules.[1–3] In vascular diseases activated leukocytes emigrate, adhere to the endothelial wall and migrate through the arterial wall, resulting in the transfer of macrophages rich in oxidized lipoproteins that trigger of onset of atherosclerotic plaque formation.[4–8]

The behavior of chemokines and soluble inflammatory markers has been investigated and current findings revealed elevated plasma concentrations of adhesion molecules in patients affected by coronary artery disease[9–16] and carotid atherosclerosis.[17,18] In contrast, not many studies have been conducted on the response of adhesion molecules and soluble inflammatory mediators in patients presenting with chronic peripheral arterial disease (PAD).[19–26] This study aimed to evaluate whether there is a qualitative or quantitative difference in adhesion molecules and selectin pathway in patients with PAD and control subjects at rest and after strenuous physical exercise (treadmill test). We believed that patients with PAD would have elevated plasma inflammation markers that further increased with exercise.

Methods

The following values were investigated:

- Interleukin 6 (IL-6) in plasma and supernatants (IL-6s) and in cells stimulated with 1 mg lipopolysaccharide (IL-6+LPS); tumor necrosis factor alpha (TFN() in plasma, supernatants and LPS-stimulated cells;
- E-selectin (Es), L-selectin (Ls) and P-selectin (Ps), VCAM-1 and ICAM-1.

Patients

The study cohort was made up of two groups: a group of 20 patients (mean age 68.4 ± 5.09 years) affected by second stage PAD (intermittent claudication) determined using ABI index (mean ABI 0.72 ± 0.12) and a control group of 20 healthy subjects (mean age 66.9 ± 6.1 years) (Table 1).

Table 1 Baseline characteristics of the study patients (mean values ± SD).

	PAD	Controls
Age (years)	68.4 ± 5.08	66.9 ± 6.1
Body mass index	27.8 ± 3.0	26.7 ± 2.2
Systolic BP (mmHg)	146.3 ± 8.09	144.6 ± 4.2
Diastolic BP (mmHg)	80 ± 8.9	77.5 ± 7.3

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Subjects presenting with clinical evidence of coronary artery disease, diabetes mellitus, chronic renal insufficiency or chronic active liver disease were not enrolled in the study. All the subjects were nonsmokers and had a BMI <30. Laboratory tests (complete blood count, urine test) were performed in all subjects and ruled out the presence of concomitant diseases. The entire study group performed a treadmill test (speed 3.5 km/h/12% slope). The test was interrupted at onset of calf muscle cramp in PAD patients, while the controls carried on for a maximum of 5 minutes, or until heart rate doubled. Pretest blood samples were removed from the anticubical vein in the arm after a short period of acclimatization in the room where the treadmill test was performed. Samples were also withdrawn at the end of the test when the patients were still standing on the treadmill.

Samples were immediately stored at −20°C until simultaneous determinations were performed on all.

Cytokine assay from mononuclear cells (PBMCs)

Mononuclear cells (PBMCs) were isolated using Boyum's method.[18] They were rapidly separated on a Ficoll-Hypaque gradient (Pharmacia Ind., Sweden) by centrifugation at 400 rates/min for 30 min at room temperature. The cells recovered on the surface were washed twice at 300 rates/min for 15 min at 4°C using a phosphate buffer (PBS) and then resuspended in RPMI 1640 (Gibco, Green Island, USA) in fetal calf serum (FCS) containing 2 ml glutamine and 50 mg/ml gentamycin.

Surface monocytes were washed three times and 1×10^6 monocytes (more than 90% of them were not specific for esterase positivity) were then incubated for 20 h at 37°C in a moist chamber containing 5% CO_2 and placed on a Petriperm hydrophobic dish (Haereus, Germany) and cultivated with and without a lipopolysaccharide phlogogenic stimulus (1 mg/ml LPS of *Escherichia coli*, Sigma Chem. Co., St. Louis, USA). The supernatant was collected after 20 h, filtered using a 0.2-μm millipore filter (Sigma) and stored at −70°C until determination of the two cytokines.

All the reagents were tested (LAL assay, Keby Vitrum, Stockholm, Sweden) prior to use to ensure they contained no LPS. Monocyte purity (90%) was assessed by cytofluorometer (Becton Dickinson, Italy) using anti-CD14 monoclonal antibodies (Leu M3, Becton Dickinson). Cell viability (95%) was determined using trypan blue exclusion test.

TNFα and IL-6 concentrations were determined using commercial kits and the ELISA method (Benden System, USA). The lowest concentrations observed were 8 pg/ml and 3.5 pg/ml for TNFα and IL-6, respectively. Cytokines were determined according to the producers' instructions and their optical density was read by an ELISA reader (HR 700 Dynatech Lab).

Soluble molecules assays

Soluble E-selectin, P-selectin, L-selectin and ICAM-1 and VCAM-1 were determined in the plasma using commercial immunoassay kits (Bender Med systems, Vienna Austria). Briefly, 50 or 100 μl of diluted samples (1:10) were added to the microwells precoated with specific monoclonal antibodies. The soluble molecules present in the samples or in the standards bound to antibodies adsorbed in the microwells. HRP-conjugated monoclonal anti-E-selectin, anti-P-selectin, anti-L-selectin and anti-ICAM-1 and VCAM-1 (100 μl) were added and bound to the molecules captured by the first antibody.

Following incubation for 2 hours, washing and removal of 100 μl of unbound enzyme conjugated anti-soluble molecules during a wash step, substrate solution reactive with HPR was added to the wells.

Colored products were formed in proportion to the amount of the different soluble molecules present in the samples. All the reactions were terminated by addition of acid solution and absorbance was measured within 30 min at 450 nm using a microtiter platter reader (Sorin Biomedica, Italy).

All samples were determined in duplicate; a standard curve was prepared from seven standard dilutions and soluble molecule concentration was determined datum to specific curve and expressed in nanograms per milliliter. The sensitivity limits were respectively: L-selectin 0.3 ng/ml, E-selectin 0.5 ng/ml, P-selectin 1.3 ng/ml, sICAM-1 0.5 ng/ml, sVCAM-1 0.9 ng/ml.

Statistical analyses

Variance analysis and ANOVA tests were performed on the differences between mean pre- and post-treadmill values observed in both groups. Statistical analysis was performed using specific software (SPSS 10.1 for Windows). Statistical significance was defined as $p < 0.05$.

Results

All the parameters investigated in the study at rest were higher in PAD patients in comparison with the controls and these parameters increased at the end of the treadmill test in both groups. The rise observed in PAD patients was more significant ($p < 0.001$ or <0.0001) than in controls ($p < 0.05$ or <0.001). (Tables 2–4, Figures 1 and 2).

Discussion

It is common knowledge that the symptoms of PAD (claudication) are caused by reduced oxygen supply to skeletal muscle after physical exercise. The exercise-induced ischemic state is also associated with altered muscle metabolism that further exacerbates the exercise impairment.[27] The resulting relative ischemia can alter the microcirculatory environment and thus influence white blood cell functions.[19,22,26] The study aim was to assess whether there were any detectable differences in patients with PAD and healthy controls, and whether these difference became more marked after physical stress-induced ischemia.

Our study detected a difference in leukocyte activation in patients with atherosclerosis compared with the healthy controls, both at rest and after exercise. In fact, our results showed higher resting levels of soluble inflammatory mediators and adhesion molecules in patients with PAD than observed in controls. This difference can be explained by the presence of vascular damage in the former, as even at rest these arterial lesions negatively influence vascular

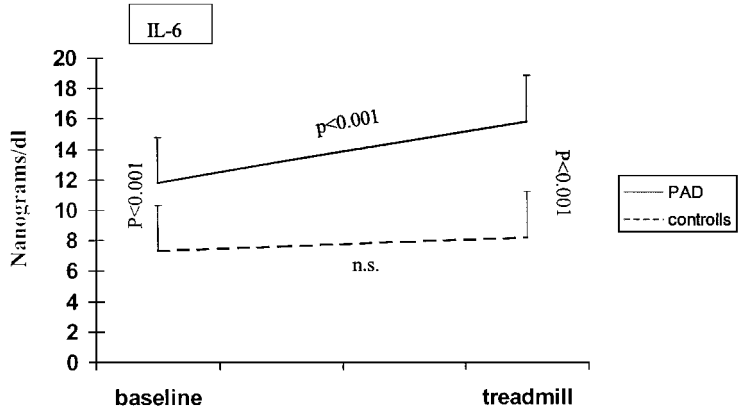

Figure 1 Interleukin 6 (IL-6) concentrations after the treadmill test in PAD patients (continual line) and in controls after 5 minutes march (dashed line). Statistical comparison between pre- and post-treadmill concentrations are shown separately for both groups. Comparison between pretreadmill values of the two study groups ($p < 0.001$) and post-treadmill concentration of the two groups are also shown (0.001)

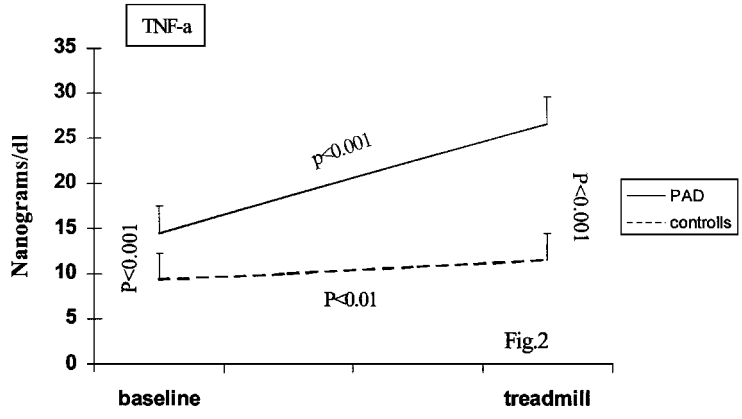

Figure 2 Post-treadmill values of tumor necrosis factor alpha (TNFα) in PAD patients ($p < 0.001$) (continual line) and in controls ($p < 0.01$) after 5 minutes march (dashed line). Statistical comparison between pre- and post-treadmill values is shown separately for the two study groups. Comparison between pretreadmill values of the two study groups ($p < 0.001$) and post-treadmill concentration of the two groups are also shown (0.001)

and arterial hemodynamics and therefore alter macrophage function in atherosclerotic patients.

Strenuous physical exercise modifies the environment in both groups of subjects and triggers activation of circulating blood cells (especially white blood cells) and their interaction with endothelial cells. The literature reported differences in the production of soluble inflammatory mediators between patients affected by peripheral atherosclerosis and healthy subjects.[19–26] However, we believe the behavior of the acute ischemia induced by the treadmill test and confirmed by onset of pain is noteworthy. Intermittent claudication occurs in compensated 2nd stage PAD patients only with exercise.[28]

We observed augmented post-treadmill plasma concentrations of soluble mediators and adhesion chemokines in both PAD patients and healthy subjects and our findings agree with other investigations on leukocyte function in atherosclerosis.[3,4,9,16] Moreover, our data confirm that blood cells, in particular white blood cells, are activated in patients affected by atherosclerosis, and revealed that the white blood cell response seems more evident in conditions of elevated hemodynamic stress presenting deficiently perfused muscle tissue stress.

We are aware of the limitations of the two protocols used in our study, which were similar regarding speed and slope, but not duration. In PAD patients the physical stress needed to perform the treadmill test may have determined transient ischemia, while in healthy patients the doubled heart rate was likely related to muscle fatigue caused by the treadmill test. Post-treadmill concentrations of cytokines, adhesion cells and selectins were elevated in both study groups.

We believe that the elevated post-treadmill concentrations of all the parameters investigated in our study reveals a close relationship between maximum physical stress and activation of white blood cells. Moreover, we suggest that increased circulation concentrations of both cytokines and adhesion molecules can determine further changes in leukocytes (rolling, adhesion) and lead to endothelial damage, thus supporting the hypothesis that white blood cells are involved in ischemia as well as the pathophysiology of vascular disorders.

Table 2 Plasma concentrations (ng/ml) of IL-6, TNFα, E-selectin, L-selectin, P-selectin, ICAM-1 and VCAM-1 in PAD patients and controls before (baseline) and after physical exercise (treadmill test).

	PAD	Controls	*p*-value
IL-6			
Baseline	11.81 ± 1.24	7.30 ± 1.16	<0.001
Treadmill	15.80 ± 2.35	8.15 ± 1.22	<0.001
TNFα			
Baseline	14.48 ± 3.32	9.32 ± 2.35	<0.001
Treadmill	26.65 ± 5.12	11.5 ± 2.45	<0.001
E-selectin			
Baseline	66.69 ± 1.21	6.55 ± 1.48	<0.0001
Treadmill	70.90 ± 1.235	8.15 ± 1.22	<0.0001
L-selectin			
Baseline	10.01 ± 3.15	5.48 ± 0.14	<0.0001
Treadmill	14.7 ± 3.52	6.56 ± 0.15	<0.0001
P-selectin			
Baseline	138.5 ± 2.08	107.3 ± 2.04	<0.001
Treadmill	176.9 ± 4.26	122.6 ± 1.75	<0.001
ICAM-1			
Baseline	316.7 ± 4.05	207.65 ± 4.98	<0.001
Treadmill	420.8 ± 10.15	262.75 ± 6.61	<0.001
VCAM-1			
Baseline	485.09 ± 14.28	464.35 ± 11.3	<0.001
Treadmill	576.16 ± 16.1	544.15 ± 11.9	<0.001

Table 3 Concentrations (ng/ml) of IL-6 and TNFα measured in supernatants in PAD patients and controls before and after physical exercise.

	PAD	Controls	*p*-value
IL-6 supernatant			
Baseline	150.53 ± 19.05	83.0 ± 7.1	<0.0001
Treadmill	484.16 ± 72.02	274.22 ± 52.10	<0.0001
TNFα supernatant			
Baseline	110.33 ± 15.5	71.38 ± 9.8	<0.001
Treadmill	21043 ± 22.8	121.30 ± 15.7	<0.001

Table 4 Concentrations (ng/ml) of IL-6 and TNFα in cells stimulated with lipopolysaccharide in PAD patients and controls before and after physical exercise.

	PAD	Controls	*p*-value
IL 6+ LPS			
Baseline	307.9 ± 46.28	210.8 ± 31.25	<0.0001
Treadmill	4920.5 ± 738.7	2450.62 ± 425.7	<0.0001
TMFα + LPS			
Baseline	1249.5 ± 83.56	748.4 ± 100.3	<0.0001
Treadmill	2320.5 ± 158.8	1426.40 ± 77.8	<0.0001

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