Inflammation and platelet activation in peripheral arterial occlusive disease

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M Montagnana, C Fava, E Arosio, et al. Inflammation and platelet activation in peripheral arterial occlusive disease. Int J Angiol 2007;16(3):84-88.

OBJECTIVES: Epidemiological evidence indicates that inflammation accompanies the progression of atherosclerosis. The aim of the present cross-sectional study was to define relationships between platelet activation and inflammation in patients with mild to severe (stages II to IV) peripheral arterial occlusive disease (PAOD) and matched controls. The effect of chronic administration of low-dose acetylsalicylic acid was investigated.

METHODS: Subjects were studied on a single occasion. C-reactive protein (CRP) and two indexes of in vivo platelet activation were measured – the urinary excretion of 11-dehydrothromboxane (TX) B₂ by immunoassay and circulating platelet-monocyte aggregates (PMAs) by flow cytometry.

Peripheral arterial occlusive disease (PAOD) of the lower limbs is a prototypical atherosclerosis-determined disease. This local reduction in arterial lumen diameter leads to chronic ischemia in a limited number of subjects while diffuse atherosclerosis, as well as coexisting cardiovascular risk factors, are determinants of major cardio- and cerebrovascular events (1).

Platelet activation is a requirement for atherothrombosis and a predictor of its occurrence (2,3). Increased platelet activation was observed in patients with effort-induced leg pain (stage II of the Lriche-Fontaine classification of PAOD) and was found to be related to coexisting cardiovascular risk factors, rather than atherosclerosis per se (4). There is evidence that the progression of PAOD is also related to inflammation (5,6). Alterations in inflammatory markers correlate independently with walking distance and other clinical indexes of functional impairment in patients with PAOD, and predict the clinical outcome of the disease (7-9). Plasma C-reactive protein (CRP), as well as other markers of inflammation, are predictors of future cardiovascular events in the general population and in subjects with cardiovascular risk factors, suggesting a causal relationship between inflammation and the progression of atherosclerosis (6,10,11). Inflammation has a central role in the pathophysiology and clinical progression of atherosclerosis, from the initial accumulation of T lymphocytes and monocytes in the subendothelium, to plaque instability and thrombotic complication (12,13). Activated

RESULTS: Plasma PMAs and urinary 11-dehydro-TXB₂ were significantly increased in PAOD patients compared with controls (P<0.01 for all). A positive correlation between 11-dehydro-TXB₂ and CRP was found in the study population (r_s=0.63, P<0.001). Using logistic regression analysis, CRP was the only independent correlate of 11-dehydro-TXB₂ (_{CRP}=11.9, P<0.01), whereas only the presence of PAOD was an independent predictor of high PMA levels (_{PAOD}=13.7, P=0.001). Chronic administration of acetylsalicylic acid reduced 11-dehydro-TXB₂, but not PMA and CRP.

CONCLUSIONS: There is evidence that platelet activation in patients with PAOD is related to the vascular disease and is dependent on the severity of inflammation.

Key Words: 11-dehydrothromboxane B₂; Acetylsalicylic acid; Inflammation; Peripheral arterial occlusive disease; Platelet activation; Platelet-monocyte aggregates

platelets participate in the development of atherosclerosis by promoting inflammation in the vessel wall and forming the thrombus (14-16). Therefore, a close relationship between inflammation and platelet activation may be hypothesized in PAOD at any stage of the disease.

The aim of the present study was to explore the relationships among the presence of overt atherosclerosis, inflammation and platelet activation in patients with mild to severe PAOD and appropriate controls.

In vivo platelet activation was quantified by measuring the urinary excretion of a major thromboxane (TX) A₂ metabolite, 11-dehydro-TXB₂, and circulating platelet-monocyte aggregates (PMAs). While urinary 11-dehydro-TXB₂ is mostly generated by the activity of prostaglandin H synthase (cyclooxygenase)-1 in platelets, PMAs derive from the interaction of activated platelets with circulating leukocytes mediated by adhesion molecules.

METHODS

Patient population

Twenty-six patients with PAOD and an equal number of subjects with no evidence of vascular disease were studied. Patients were recruited consecutively among those admitted to the Vascular Rehabilitation Division of the University of Verona, (Verona, Italy). The severity of the disease was defined according to the Lriche-Fontaine classification; therefore, patients with PAOD stages II to IV were included.

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Control volunteers were affiliates of a veterans' organization and their relatives. Patients and controls were matched for age (within five years) and sex.

Diagnosis of PAOD was confirmed or excluded by clinical evaluation and Doppler ultrasound measurement of the ankle-arm index (ie, the ratio of ankle to arm systolic blood pressure), performed by trained physicians according to a standard protocol (17). Exclusion criteria for all the studied subjects included surgery within the previous two months, and infective disease, malignancy, or any acute or chronic inflammatory disorder. On a single occasion, urine samples were collected from each subject for the measurement of urinary excretion of 11-dehydro-TXB₂ and creatinine. Blood samples for the measurement of circulating PMAs and a glycometabolic profile were also collected. A clinical examination and hemodynamic tests were also performed.

Of the 26 PAOD patients, nine patients were not receiving any antiplatelet medication and 17 patients were taking ticlopidine at a dose of 250 mg twice per day. Because it is well established that acetylsalicylic acid (ASA) inhibits TXA₂ formation, patients on chronic pharmacological treatment with ASA were excluded.

Twenty-three PAOD patients were on antihypertensive medication – 10 received statins, 14 received either oral antidiabetic drugs or insulin, and one patient received both types of medication.

The control subjects had no signs or clinical evidence of PAOD, and no personal history of cardiovascular events. Also, control subjects were studied without modifying their current treatments – 10 were taking antihypertensive drugs and two were taking statins. None were taking antidiabetic drugs.

Demographic, anthropometric and biochemical characteristics of patients with PAOD (mean $\pm$ SD) age 71.3 $\pm$ 8 years, range 58 to 84 years; 15 men) and matched controls (69.3 $\pm$ 8 years, range 60 to 85 years; 15 men) are presented in Table 1.

To further explore the relationship between inflammation and platelet activation in patients treated with ASA, 14 PAOD patients chronically treated with ASA (100 mg to 300 mg once per day) were added to the patient population, along with 14 healthy controls matched for gender and age. Therefore, a total of 40 patients and 40 controls were studied.

Diabetes mellitus was defined in accordance with the criteria of the American Diabetes Association (18). Hypertension was defined as a systolic or diastolic blood pressure of 140/90 mmHg or greater, or current treatment with antihypertensive drugs (19). Hypercholesterolemia was defined as either low-density lipoprotein cholesterol greater than 3.36 mmol/L or the use of hypocholesterolemic drugs. Subjects were considered to be smokers if they smoked more than five cigarettes per day during the previous year.

Blood pressure was recorded (the mean of three readings) using an automatic device (Dinamap, GE Healthcare, Finland) with the subjects supine for at least 15 min; brachial pulse pressure was calculated. Body mass index was measured in kg/m².

The present study was approved by the Ethics Committee of the Verona University Hospital (Verona, Italy). Written informed consent was obtained from all subjects.

Flow cytometry assay of circulating platelet-monocyte complexes

Peripheral blood was collected by venipuncture, without venous stasis, using K₂EDTA for anticoagulation. Samples for

TABLE 1
Demographic and biochemical characteristics of patients with peripheral arterial occlusive disease (PAOD) and control subjects

Characteristic	PAOD patients (n=26)	Controls (n=26)
BMI, kg/m ²	26.8 $\pm$ 4.6	25.3 $\pm$ 3.7
Diabetes mellitus, n	14*	4
Hypertension, n	23*	14
Cigarette smoking, n	1**	4
Hypercholesterolemia, n	14	12
SBP, mmHg	144.8 $\pm$ 18.3	140.6 $\pm$ 16.9
DBP, mmHg	77.5 $\pm$ 9.4	81.7 $\pm$ 11.6
HDL cholesterol, mmol/L	1.3 $\pm$ 0.4	1.5 $\pm$ 0.6
LDL cholesterol, mmol/L	2.8 $\pm$ 0.9	3.0 $\pm$ 0.9
Triglycerides, mmol/L	1.7 $\pm$ 1.2	1.8 $\pm$ 1.0
Creatinine, μ mol/L	76.9 $\pm$ 22.1	91 $\pm$ 41.5
Glucose, mmol/L	6.1 $\pm$ 1.5	6.1 $\pm$ 2.2

Data are expressed as mean $\pm$ SD unless stated otherwise. **P<0.05 versus controls; *P<0.01 versus controls. BMI Body mass index; DBP Diastolic blood pressure; HDL High-density lipoprotein; LDL Low-density lipoprotein; SBP Systolic blood pressure

the flow cytometry assay were immediately incubated for 60 min with ThromboFix Platelet Stabilizer (Beckman Coulter Inc, USA).

Circulating PMA was quantified using dual-label flow cytometry according to the following procedure. Blood samples (30 μ L) were incubated in the dark at room temperature for 15 min with 10 μ L of anti-CD14-PC5 (a lipopolysaccharide receptor; Beckman Coulter Inc, USA) and 20 μ L of anti-CD61-FITC (glycoprotein IIIa; Beckman Coulter Inc, USA). Isotype-matched mouse immunoglobulin G FITC (Beckman Coulter Inc, USA) was used as a negative control. Red cells were lysed in the dark for 10 min by adding 1.5 mL of KHCO₃ (1 g/L) and NH₄Cl (8.26 g/L) in water. Flow cytometry was performed with a Coulter Elite FACSscan (Coulter Corporation, USA), with data handling by Expo 32 ADC Cytometer software (Beckman Coulter Inc, USA). A minimum of 2000 CD14+ events were acquired. Platelet-monocyte interactions were assessed using an immunological gate to measure the number of events with double positivity for CD14-PC5 (used to identify monocytes) and CD61-FITC (used to identify platelets). Preliminary experiments showed that under these conditions, a limited number of P selectin-expressing platelets (CD61+/CD62+) were present in PMAs (less than 2%). Fixation was required, because preliminary experiments showed a time-dependent increase in the number of PMAs (CD14+/CD61+) and CD61+/CD62+ positive platelets in nonfixed blood samples.

The intra-assay and interassay coefficients of variability in the flow cytometry measurement of PMAs were 8% and 13%, respectively (n=6).

Measurement of urinary 11-dehydro-TXB₂

The urinary excretion of 11-dehydro-TXB₂ was evaluated from overnight urine collections (from 12:00 to 08:00 on the following day). Samples from timed urine collections were immediately centrifuged (at 1500 g for 10 min) and then stored at –80°C. To evaluate the percentage of recovered material at the

TABLE 2
Determinants of the urinary excretion of 11-dehydro-thromboxane (TX) B₂ and platelet-monocyte aggregate (PMA) by multivariate logistic regression analysis (n=52)

	11-dehydro-TXB ₂			PMA		
	Exp (B)	95% CI	P	Exp (B)	95% CI	P
Presence of PAOD	—	—	—	13.7	1.7–110	0.001
hs-CRP >3.1 mg/L	11.9	2.15–66.2	0.005	—	—	—

Other variables discarded from the models: age, hypertension, hypercholesterolemia, smoking habit, diabetes, stage of peripheral arterial occlusive disease (PAOD) (stage II to III for PAOD patients, stage I for controls). Exp (B) Exponent (B); hs-CRP Highly sensitive C-reactive protein

end of the purification procedure, a small amount of [³H]TXB₂ (8000 decays/min) was added to the urine samples (10 mL). After adjusting the urine pH to between 3.5 and 4.0 with formic acid, prostanoids were extracted using C₁₈ cartridges (HF Bond Elut LRC, Varian Inc, USA). The fraction containing 11-dehydro-TXB₂ was eluted with 10 mL of ethyl acetate. After evaporation to dryness, the extracts were reconstituted with 0.15 mL of ethanol for reverse-phase high performance liquid chromatography separation. This was performed as follows: 20 µL of the reconstituted extracts were injected into a C₁₈ column (15 cm × 4.6 mm, 5 µm film thickness; Supelco Analytical, USA) and eluted with a solvent system consisting of an acetonitrile to H₂O ratio of 3:7 volume/volume, acidified with glacial acetic acid (pH 3.5 to 4.0) at a constant flow rate of 1 mL/min. The fraction containing 11-dehydro-TXB₂ was collected, evaporated to dryness and reconstituted with 1 mL of assay buffer. The recovery at the end of the purification procedure was determined for each individual sample by measuring the amount of eluted [³H]TXB₂ and was found to be 64.4%±13.3% (n=80). 11-dehydro-TXB₂ was measured in the reconstituted high performance liquid chromatography eluates using a commercial enzyme immunoassay kit (Cayman Chemical, USA) following the standard procedure recommended by the manufacturer, which includes a prolonged incubation in enzyme immunoassay buffer (2 M diethanolamine, pH 9.0, containing 10 mM EDTA, 4 M sodium chloride, 1% bovine serum albumin and 0.1% sodium azide) that is designed to allow conversion of all the 11-dehydro-TXB₂ into one conformation.

Biochemical analyses

Blood samples were collected to determine plasma concentration of glucose, total and high-density lipoprotein cholesterol, triglycerides and creatinine. Plasma was prepared from blood collected in tubes containing lithium heparin by centrifugation. The concentration of serum highly sensitive CRP (hs-CRP) was determined using a highly sensitive latex-enhanced immunoturbidimetric assay with a detection limit of 0.1 mg/L on a Dade Behring Nephelometer (Dade Behring GmbH, Germany). Serum and plasma samples were stored at –80°C. All analyses were performed in a certified laboratory.

Statistical analysis

Data were tested for normality by a Kolmogorov-Smirnov goodness-of-fit test. The Kruskal-Wallis test and the Mann-Whitney U test were used when appropriate. Bonferroni's correction was applied to post hoc analysis. The Spearman coefficient (r_s) was calculated to quantify the correlation

between variables. Multivariate logistic regression analysis was used for the OR estimates, performing backward stepping of variables, with preassigned P-values equal to 0.05 controlling the stepping removal. The model was evaluated using a goodness-of-fit χ^2 test. All calculations were done with SPSS software (version 12.0.1, SPSS Inc, USA). Multivariate logistic regression analysis, in which high or low urinary excretion of 11-dehydro-TXB₂ (above, or equal to or below 1451.3 pg/mg creatinine, the median value) and high or low PMA levels (above, or equal to or below 30.2%, the median value) were the dependent variables, was performed to explore which independent variables were correlated with indexes of platelet activation. Any P<0.05 was considered statistically significant. Continuous variables are presented as mean ± SD in the text and tables. Discrete variables are presented as counts. Data are presented as box and whiskers diagrams in Figure 1 (median and interquartile intervals).

RESULTS

As expected on the basis of selection criteria, the prevalences of both diabetes mellitus and hypertension were higher in patients with PAOD than in the matched controls (Table 1). Systolic and diastolic blood pressure values were similar in patients and matched controls. Differences in platelet activation and hs-CRP were observed between patients and matched controls (P<0.01 for both, Figure 1A).

Differences were also observed in serum hs-CRP (Figure 2) when patients with PAOD were divided according to the presence or absence of leg ischemia at rest (stages III and IV versus stage II of the Leriche-Fontaine classification), in that more elevated values were observed in patients with stage III and stage IV vascular disease (Figure 1B). Patients with leg ischemia at rest had higher levels of 11-dehydro-TXB₂, but not of PMA (P<0.05 for 11-dehydro-TXB₂, but not for PMA) (Figure 1B).

When PAOD patients were grouped according to ticlopidine treatment (17 treated versus nine nontreated patients), no difference in 11-dehydro-TXB₂ (2692.1±1476.4 ng/mg versus 2664.8±2102.4 ng/mg creatinine; P>0.05), PMA (43.2%±12.1% versus 40%±8.5%; P>0.05) or hs-CRP (7.2±5 mg/L versus 7.3±6.5 mg/L; P>0.05) was found.

The studied indexes of platelet activation were related to inflammation. In particular, 11-dehydro-TXB₂ and hs-CRP correlated positively in PAOD patients, as well as in the whole study population (Figure 2). A weaker correlation was also observed between PMA and hs-CRP in the whole population (r_s=0.30, P<0.05), as well as between the two indexes of platelet activation (r_s=0.33, P<0.05).

In a multivariate logistic regression analysis, the presence of a high value of hs-CRP (greater than 3.1 mg/L) was the only independent variable correlated to high TXA₂ metabolite excretion (Table 2). The presence of PAOD was the only independent predictor of high PMA levels (Table 2).

In the analysis of PAOD patients chronically treated by ASA, it was then observed that the 11-dehydro-TXB₂ was significantly lower in patients treated with ASA than in PAOD patients not treated by ASA and matched controls. PMA and hs-CRP levels were higher in PAOD patients with respect to controls, but irrespective to ASA treatment (Table 3).

DISCUSSION

The present study was designed to investigate the independent contribution of inflammation and overt atherosclerosis to

Figure 1) Platelet-monocyte aggregate (PMA) and highly sensitive C-reactive protein (hs-CRP) levels in peripheral arterial occlusive disease (PAOD) patients and matched controls (A), and in different Leriche-Fontaine stages (B). 11-dh-TXB₂ 11-dehydrothromboxane B₂; NS Not significant

Figure 2) Correlation between highly sensitive C-reactive protein (hs-CRP) and 11-dehydrothromboxane B₂ (11-dh-TXB₂). Dark bullets represent peripheral arterial occlusive disease (PAOD) patients, and empty bullets represent controls

TABLE 3
Demographic and biochemical characteristics of patients with peripheral arterial occlusive disease (PAOD) and control subjects

Characteristic	PAOD patients with ASA (n=14)	PAOD patients without ASA (n=14)	Controls (n=14)
Age, years	72.5±8	73.6±8.7	73.3±8.2
Sex, male	9	9	9
Stages III and IV of PAOD, n	5	5	—
hs-CRP, mg/L	8.1±7.3*	6.6±4.6**	2.9±2.6
PMA, %	40.9±12.8*	42.3±11.9**	33.3±12.4
Urinary 11-dehydro-TXB ₂ , pg/mg creatinine	313.3±160.7***†	2716.1±1647.4*	1204.9±659.8

Data are expressed as mean ± SD unless otherwise stated. *P<0.05 versus controls; **P<0.01 versus controls; ***P<0.001 versus controls; †P<0.001 versus PAOD patients without acetylsalicylic acid (ASA). hs-CRP Highly sensitive C-reactive protein; PMA Platelet-monocyte aggregate; TX Thromboxane

in vivo platelet activation. Patients with PAOD and pair-matched subjects without clinical evidence of PAOD were studied.

The major finding of the present study is that platelet activation, assessed by measuring urinary excretion of 11-dehydro-TXB₂, is related to the presence and severity of PAOD and to inflammation.

Experimental evidence indicates that inflammation may be the trigger for platelet activation and thrombus formation (20). Atherosclerosis is an inflammatory disease (12,13). A mild degree of inflammation is associated with cardiovascular risk factors, which predicts cardiovascular events in the general population. Increased levels of inflammation markers, including hs-CRP and interleukin-6, have recently been demonstrated to be a predictor of the progression of peripheral artery disease in large population-based studies (5,7,8).

Previous cross-sectional studies showed that the severity of the inflammatory state predicts mortality in patients with more advanced peripheral artery disease (6,10,11). However, both the burden of atherosclerosis and the severity of inflammation within vascular lesions, and possibly systemic inflammation, are determinants of the thrombotic complication of atherosclerosis. In fact, in patients with unstable angina and PAOD, plasma CRP levels largely exceed those observed in patients with peripheral atherosclerotic lesions. They do not exceed those observed in myocardial ischemia, indicating that the release of proinflammatory compounds accompanies, and is implicated in, acute thrombotic events (21). We observed that inflammation accompanies the development of the disease in subjects with PAOD, with the most elevated values of hs-CRP in patients with severe disease (ie, pain at rest or ischemic ulcers).

In a previous study (4), the urinary excretion of 11-dehydro-TXB₂ was found to increase in patients with PAOD compared with controls. In addition, patients who subsequently developed a major cardiovascular event had more elevated TX metabolite excretion than those who remained event-free. Consistent with that observation, we found that the urinary excretions of both 11-dehydro-TXB₂ and plasma PMA were higher in PAOD patients than in controls. However, only urinary 11-dehydro-TXB₂ gradually increased with the severity of the disease and correlated with CRP levels. PMAs were only weakly related to inflammation, although under experimental conditions, PMAs are generated by the interaction of stimulated platelets expressing P selectin with quiescent or activated monocytes (22,23).

The observed correlation between 11-dehydro-TXB₂ and CRP prompted us to further explore the relationship between platelet activation and inflammation by investigating a group of PAOD patients chronically treated with low doses of ASA. We found that, despite a marked reduction in TX metabolite excretion, the index of substantial inhibition in platelet TXA₂ generation (24). hs-CRP was significantly higher in PAOD patients than in untreated subjects, suggesting that inflammatory

events precede platelet activation and that low-dose ASA does not alter the inflammatory response (24-26). In addition, the apparent lack of efficacy of PMA formation confirms that platelet activation is partly insensitive to cyclo-oxygenase inhibition.

CONCLUSION

We observed that increased platelet activation in patients with PAOD is predicted by inflammation that characterizes this clinical condition. Further mechanistic investigation on the signals linking inflammation to platelet activation may offer clues for more effective prevention of cardiovascular events.

ACKNOWLEDGMENTS: The present study was supported by grants given to Dr Alessandro Lechi by Region Veneto, and to Dr Clara Lechi Santonastaso by the Italian Ministry of University and Research (prot 2004064152_003).

CONFLICT OF INTEREST STATEMENT: All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence the work.

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