Leukocytes and complement in atherosclerosis

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c. Monocyte CD11b expression is a predictor of future vascular events in patients with coronary artery disease

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ABSTRACT

Introduction: Although, leukocyte activation has been linked to atherogenesis, the available in vivo evidence for its role in the progression of atherosclerosis is scarce. In this prospective follow-up study, we evaluated the predictive value for future cardiovascular (CV) events of leukocyte activation markers, inflammatory variables and classical CV risk factors in patients with and without coronary artery disease (CAD).

Methods: Patients undergoing coronary angiography were included consecutively and were divided in two groups consisting of subjects without (CAD-; n=26) and with (pre-existent) CAD (CAD+; n=67). The expression of monocyte and neutrophil CD11b and neutrophil CD66b was determined by flowcytometry.

Results: No differences were found between the groups for leukocyte activation markers, CRP, leukocyte and platelet counts. During follow-up of (mean±SEM) 2.0±0.03 yrs, 20 CV events occurred in the CAD+ patients and one in the CAD- group. Univariate analysis showed that CAD, gender, systolic blood pressure, TG, LDL-C, platelet counts, monocyte CD11b expression, and the use of statins and aspirin were significantly associated with a future event. Multiple logistic regression analysis demonstrated that monocyte CD11b expression (odds ratio (OR)=1.08 (1.01-1.14)), pre-existing CAD (OR=9.08 (0.95-86.37)) and systolic blood pressure (OR=1.04 (1.01-1.08)) were determinants of CV events. After exclusion of the CAD- group, monocyte CD11b expression remained the only predictor of CV events in the CAD+ group (OR=1.07 (1.01-1.14)).

Conclusions: Monocyte CD11b is a determinant for risk of future cardiovascular events during a follow-up of two years. These data emphasize the role of monocyte activation in the progress of atherosclerosis.

INTRODUCTION

In the past few years much attention has been paid to markers predicting future cardiovascular events. Since atherosclerosis is an inflammatory disease (1,2), the focus is mostly on markers of inflammation. C-reactive protein (CRP) (3-6), leukocyte counts (7) and complement component 3 (C3) (8) have been frequently associated with the risk of future cardiovascular events.
Leukocyte activation has been described as one of the crucial steps in atherogenesis (1). The expression of leukocyte integrins like neutrophil and monocyte CD11b, and neutrophil CD66b have been linked directly (9-11), and indirectly (12-16), to atherosclerosis. CD11b (also termed MAC-1 or CR3) is involved in early adhesion of leukocytes to the endothelium (17,18). CD66b (also termed CEACAM8) is a degranulation marker of neutrophils (19).
To the best of our knowledge, no studies have been published investigating leukocyte activation markers CD11b and CD66b as possible indicators for future vascular events. In this prospective cohort study, we aimed to evaluate whether these activation markers can be useful tools for risk prediction in a population of patients with and without CAD, under standard cardiovascular therapy.

MATERIALS AND METHODS

Subjects

Subjects who visited the outpatient clinic of the Department of Cardiology of the Sint Franciscus Gasthuis, Rotterdam, the Netherlands, and who were scheduled to undergo a diagnostic coronary angiography were asked to participate. These patients were selected consecutively and their CAD status was unknown at the time of inclusion.
Exclusion criteria were: The presence of inflammatory disorders, e.g. rheumatoid arthritis, systemic lupus erythematosus and infections, CRP>10 mg/L, disorders of kidney, liver and thyroid function. Only postmenopausal women were included based on a history of secondary amenorrhea of at least 1 year and not using hormone replacement therapy.
The Institutional Review Board of the St. Franciscus Gasthuis Rotterdam and the regional independent medical ethics committee of the Maasstad Hospital Rotterdam approved the study. All participants gave written informed consent.

Study design

The study was designed as a prospective follow-up study. Subjects were divided into 2 groups, based on the outcome of the angiography. The first group consisted of patients who had neither coronary atherosclerosis, nor a history of peripheral or cerebral vascular disease

(CAD- patients). Their indication for coronary angiography was the clinical presentation with typical chest pain and a positive cycle ergometry. Patients who had at least wall irregularities in one of the coronary arteries were selected for the second group (CAD+ patients).
Shortly before the angiography, venous blood was obtained from a peripheral vein of the forearm. Coronary angiography images were scored by an independent cardiologist.
The primary outcome was defined as any vascular event or any vascular intervention, evaluated by an independent investigator at approximately two years follow-up. This end point included any death of presumed vascular origin (fatal stroke, fatal myocardial infarction, sudden death, other vascular death), nonfatal stroke, nonfatal myocardial infarction, hospitalization due to stable or unstable angina pectoris and any arterial vascular intervention that had not already been planned at the time of inclusion (e.g., carotid surgery or angioplasty/stenting, coronary bypass, percutaneous coronary intervention, peripheral vascular surgery or angioplasty/stenting).

Analytical methods

All clinical chemistry measurements were performed on the same day as the diagnostic coronary angiography. Basic parameters for renal and liver function as well as glucose, CRP, total cholesterol, HDL cholesterol and TG were determined using a Synchron LX analyzer (Beckman Coulter, Brea CA, USA) according to standard procedures in our laboratory for clinical chemistry. LDL cholesterol values were calculated using the Friedewald formula. Plasma apolipoprotein (apo) AI and apoB were determined by rate nephelometry using IMMAGE with kits provided by Beckman (Beckman Coulter, Brea CA, USA). Blood cell counts were determined using the LH analyzer (Beckman Coulter, Miami FL, USA). The leukocyte differentiation was determined as a five-part differentiation on the same instruments.

Leukocyte activation markers

Blood samples for the measurement of leukocyte activation markers were collected in EDTA and were determined by flowcytometry on the same day. In order to differentiate leukocytes in lymphocytes, monocytes and neutrophils a CD45 (Immunotech Coulter, Marseille, France) versus SS gating strategy was used. Lymphocytes were defined as CD45 positive and low sideward scatter. Monocytes were defined as CD45 positive and intermediate sideward scatter. Neutrophils were defined as CD45 weak and high sideward scatter. The gates were set quite narrow for optimal differentiation of these cell populations rather than for completeness. For tube 1 twenty μL blood from an EDTA-anti-coagulated blood sample was added to 2.5 μL of each CD66b FITC (Immunotech Coulter, Marseille, France), CD11b PE (Immunotech Coulter, Marseille, France) and CD45 ECD (Immunotech Coulter, Marseille, France). Cells were incubated for 15 minutes in the dark at room temperature. Erythrocytes were lysed by adding 300 μL of ice-cold isotonic erythrocyte lysing solution (NH4Cl 0.19M; KHCO3 0.01M; Na2EDTA•2H2O

0.12M, pH 7.2) for 15 minutes. A Coulter Epics XL-MCL flowcytometer with a 488nm Argon ion laser and EXPO 32 software was used for measurement and analysis. Cells were acquired during 2 minutes per sample. On average a total of 25.000 leukocytes per sample were measured. Fluorescence intensity of each cell was expressed as the mean fluorescence intensity (MFI), given in arbitrary units (AU). Additional experiments (data not shown) did not show significant differences between EDTA and heparin anti-coagulated blood for CD11b and CD66b expression. Furthermore we also did not find significant differences of CD11b and CD66b expression in a protocol in which we did not use ammoniumchloride for erythrocyte lysis (data now shown) .

Statistical analysis

Data are given as mean±SEM in the text, Tables and Figures. Baseline differences between the groups were tested with independent Student's t-tests. The prevalence of CAD, medication use, smoking behavior and the prevalence of type 2 diabetes mellitus were tested by Chi-square tests. Binary logistic regression was used to investigate the impact of various predictive variables for CAD events using a two step procedure. In the first step, we used univariate analysis to compare the impact of baseline variables on CAD events. In the second step, each variable reaching $P<0.10$ in the univariate analysis was included in the (backward stepwise) multiple logistic regression. Since CAD+ patients are older (natural course), the variable age was forced into the analysis. Interaction effects with age and CAD were evaluated. Data were analyzed in SPSS 16.0. P-values < 0.05 (2-tailed) were considered statistically significant.

RESULTS

Baseline characteristics (Tables 1 & 2)

A total of 93 subjects were included of whom 26 were CAD- subjects and 67 CAD+ patients. In the follow-up period, one of the patients in the CAD+ group died due to a malignancy and another one was lost to follow-up. Therefore, both were excluded and the analysis was carried out for 26 CAD- and 65 CAD+ patients.

The baseline characteristics of the two groups are listed in Table 1. CAD+ patients were older and had lower diastolic blood pressure, as well as lower total cholesterol, LDL and total apoB than CAD- patients. CAD- patients had higher apoAI when compared to CAD+ patients, with a trend for HDL. There was trend for higher waist circumference in CAD+ patients. The other cardiovascular risk factors did not differ between the groups.

There were significantly less women in the CAD+ group than in CAD- group (Table 2). CAD+ patients used more statins and aspirin than CAD- patients. The other variables did not differ between the groups (Table 2).

Table 1. Baseline characteristics in 26 CAD- and 65 CAD+ patients.

	CAD- (n=26)	CAD+ (n=65)	P-value
Age (years)	58.27 (2.09)	66.31 (1.32)	*0.002*
BMI (kg/m2)	27.43 (1.08)	27.58 (0.51)	0.89
Waist circumference (m)	1.00 (0.04)	1.07 (0.02)	0.07
Systolic BP (mm Hg)	137 (4)	146 (3)	0.09
Diastolic BP (mm Hg)	85 (3)	79 (1)	*0.02*
Glucose (mmol/L)	6.60 (0.41)	6.83 (0.20)	0.57
Cholesterol (mmol/L)	5.31 (0.20)	4.61 (0.14)	*0.006*
HDL-C (mmol/L)	1.35 (0.07)	1.21 (0.04)	0.07
LDL-C (mmol/L)	3.31 (0.17)	2.57 (0.13)	*0.001*
TG (mmol/L)	1.46 (0.14)	1.83 (0.13)	0.11
ApoAI (g/L)	1.51 (0.06)	1.36 (0.04)	*0.04*
ApoB (g/L)	1.08 (0.06)	0.92 (0.03)	*0.03*
CRP (mg/L)	3.04 (0.54)	2.85 (0.29)	0.74
Leukocyte counts (10^9 cells/L)	7.29 (0.41)	7.31 (0.21)	0.96
Monocyte counts (10^9 cells/L)	0.57 (0.03)	0.61 (0.02)	0.34
Neutrophil counts (10^9 cells/L)	4.57 (0.35)	4.50 (0.17)	0.83
Lymphocyte counts (10^9 cells/L)	1.99 (0.13)	1.98 (0.08)	0.91
Platelet counts (10^9 cells/L)	236 (12)	228 (7)	0.54
Complement component 3 (g/L)	1.23 (0.06)	1.23 (0.03)	0.95
Complement component 4 (g/L)	0.24 (0.01)	0.24 (0.01)	0.99
Monocyte CD11b expression (AU)	36.53 (2.42)	33.51 (1.31)	0.24
Neutrophil CD11b expression (AU)	32.32 (2.05)	30.43 (1.36)	0.17
Neutrophil CD66b expression (AU)	6.59 (0.47)	7.35 (0.29)	0.45

Data are mean (SEM). BP: blood pressure

Table 2. Gender distribution, prevalence of type 2 DM (%), smoking behavior (%) and use of medication (%) in 26 non-atherosclerotic (CAD-) and 65 CAD+ patients.

	CAD- (n=26)	CAD + (n=65)	P-value
Gender (% women)	61.5	29.2	*0.005*
Type 2 diabetes mellitus	23.0	32.3	0.27
Smoking	19.2	12.3	0.29
Statins	42.3	80.0	*0.001*
Aspirin	34.6	81.5	*<0.001*
Beta blockers	50.0	56.9	0.35
Diuretics	34.6	26.2	0.29
Ace-inhibitors	34.6	30.8	0.45
Angiotensin II receptor blockers	15.4	24.6	0.25
Calcium channel antagonists	19.2	35.4	0.10
Long-acting nitrates	7.7	16.9	0.22
Ezetimibe	3.8	9.2	0.36
Metformin	7.7	16.9	0.22
SU-derivates	3.8	13.8	0.16

Prospective analysis (Table 3)

The mean±SEM duration of follow-up was 2.0±0.03 yrs (range: 1.3 to 2.5 yrs) and did not differ between the CAD- and CAD+ groups. One patient in the original CAD- group developed an ischemic stroke (event rate: 1/26 (0.039), 95%CI: (0.001–0.196)). There were twenty events in the CAD+ group (event rate: 20/56 (0.357), 95%CI: (0.234–0.450)). Six patients were hospitalized due to stable or unstable angina pectoris (AP) and were treated by medication. Eight patients developed unstable AP and needed intervention (primary coronary angioplasty). Ischemic stroke occurred in 2 patients. Four patients were hospitalized due to ischemic heart failure of whom one underwent a CABG, two patients were treated with medication and one died.

Univariate analysis (first step) of each of the variables in Tables 1 and 2 showed that CAD status, gender, systolic blood pressure, TG, LDL, platelet counts and monocyte CD11b expression as well as the use of statins and aspirin were positively or negatively significantly associated with a future event.

These variables were used in the multiple logistic regression model in the second step to determine the variables predicting an event in the whole group and in CAD+ subgroup (Table 3). For the total group, CAD at baseline predicted an event after 2.0±0.03 years. Moreover, systolic blood pressure and monocyte CD11b expression were positively associated with a future event. LDL was negatively associated with a future event. The patients who developed an event had higher monocyte CD11b expression at baseline when compared to subjects who were event free during the two years of follow-up (38.22±3.02 vs. 31.48±1.26 AU, P=0.02).

Carrying out the analysis for the CAD+ subgroup, only leukocyte counts, LDL and monocyte CD11b expression were significant predictors of vascular events in univariate analysis. Analysis by multiple logistic regression of these variables showed that only monocyte CD11b expression predicted a future cardiovascular event (Table 3). No variables predicted future events in CAD- patients. Age was not a predictor of a vascular event for the total group, nor for the CAD+ and CAD- subgroups (Table 3).

Table 3. Variables predicting cardiovascular events in the total group and in CAD+ subgroup after multiple logistic regression.

	Total group		CAD+ patients	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Monocyte CD11b expression (AU)	1.08 (1.01-1.14)	0.02	1.07 (1.01-1.14)	0.02
Age (years)	0.97 (0.91-1.04)	0.33	1.04 (0.97-1.10)	0.27
CAD (yes)	9.08 (0.95-86.37)	0.06	-	-
Systolic BP (mm Hg)	1.04 (1.01-1.08)	0.01	-	-
LDL-C (mmol/L)	0.47 (0.23-0.98)	0.04	1.02 (0.29-1.17)	0.13

OR= Odds ratio. No variables predicted future events in CAD- patients. Age was a variable 'forced' into the analysis. BP: blood pressure.

DISCUSSION

In this study, we show for the first time that monocyte CD11b expression is an independent determinant of risk for future cardiovascular events in a randomly chosen group consisting of patients with and without CAD under standard cardiovascular medication during a follow-up of two years. This leukocyte activation marker was the only valuable predictor of events in patients with pre-existent CAD. This finding is surprising since baseline values of leukocyte activation markers were not different between patients with and without CAD. Monocyte CD11b expression, however, was higher in patients who eventually developed an event. Of the classical cardiovascular risk factors systolic blood pressure showed to be the only positive predictor of future cardiovascular events.

As mentioned before leukocyte activation, and especially CD11b expression has been linked to atherosclerosis (9-11,13-16). The fact that monocyte CD11b expression, but not the other markers, was of predictive value for vascular events, suggests that monocyte adherence to the endothelium rather than neutrophil-endothelium adherence and degranulation, may be more relevant in the progression of atherosclerosis in patients under standard treatment. In fact, no other inflammatory factors measured in our study were predictive for future events, while CRP (3-6), leukocyte counts (7) and C3 (8) have been proven as valuable determinants. It should be emphasized that we did not measure high sensitive CRP. The above mentioned hypothesis on monocyte-endothelium adhesion is strengthened by the fact that monocyte CD11b was the only predictor for events in patients with pre-existing CAD.

Previous studies showed that LDL is a positive predictor for cardiovascular events (20,21). These studies were conducted in patients not using lipid lowering medication. Interestingly, our study group consisted of patients under standard lipid lowering therapy, and LDL turned out to be a negative predictor of events. The CAD+ patients had the lowest fasting LDL at baseline. Presumably, those patients had been treated more aggressively, probably because of their higher risk to develop clinical complications. Therefore, LDL was a confounding variable in our analysis.

Apart from HDL and apoAI, the baseline lipids and diastolic blood pressure were more favorable in CAD+ subjects. This is not surprising, since these patients used more cardiovascular medication.

The limited number of patients included is a potential limitation in our study. However, we believe that this study provides enough support for monocyte CD11b expression to be, at least a potential determinant for future vascular events in high risk patients.

Finally, we did not determine the degree of activation of the integrins. However, the expression of Mac-1 (CD11b) is a good marker for the rate of its activation. The activation of the integrin Mac-1 (8B2 neoepitope) occurs rapidly after coronary stenting, followed by CD11b expression (22). Upregulation of CD11b is paralleled by the activation of its integrin.

In conclusion, in comparison to classical cardiovascular risk factors such as lipids, leukocyte counts and CRP, monocyte CD11b expression is the only determinant of future vascular events in patients with pre-existent CAD under standard cardiovascular medication during a follow-up of two years.

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Disclosures
None declared.

REFERENCES

1. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115–26
2. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. Nat Rev Immunol 2006;6:508–19.
3. Willerson JT, Ridker PM. Inflammation as a cardiovascular risk factor. Circulation 2004;109:II2–10.
4. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, Lowe GD, Pepys MB, Gudnason V. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. N Engl J Med 2004;350:1387–97.
5. Mora S, Rifai N, Buring JE, Ridker PM. Additive value of immunoassay-measured fibrinogen and high-sensitivity C-reactive protein levels for predicting incident cardiovascular events. Circulation 2006; 114:381–7.
6. Folsom AR, Chambless LE, Ballantyne CM, Coresh J, Heiss G, Wu KK, Boerwinkle E, Mosley TH Jr, Sorlie P, Diao G, Sharrett AR. An assessment of incremental coronary risk prediction using C-reactive protein and other novel risk markers: the atherosclerosis risk in communities study. Arch Intern Med 2006; 166:1368–73.
7. Madjid M, Awan I, Willerson JT, Casscells SW. Leukocyte count and coronary heart disease: implications for risk assessment. J Am Coll Cardiol 2004;44:1945–56.
8. Muscari A, Massarelli G, Bastagli L, Poggiopollini G, Tomassetti V, Drago G, Martignani C, Pacilli P, Boni P, Puddu P. Relationship of serum C3 to fasting insulin, risk factors and previous ischaemic events in middle-aged men.Eur Heart J 2000;21:1081–90.
9. Berliner S, Rogowski O, Rotstein R, Fusman R, Shapira I, Bornstein NM, Prochorov V, Roth A, Keren G, Eldor A, Zeltser D. Activated polymorphonuclear leukocytes and monocytes in the peripheral blood of patients with ischemic heart and brain conditions correspond to the presence of multiple risk factors for atherothrombosis. Cardiology 2000;94:19-25.
10. Mazzone A, De Servi S, Mazzucchelli I, Fossati G, Gritti D, Canale C, Cusa C, Ricevuti G. Increased expression of CD11b/CD18 on phagocytes in ischaemic disease: a bridge between inflammation and coagulation. Eur J Clin Invest 1997;27:648-52.
11. De Servi S, Mazzone A, Ricevuti G, Mazzucchelli I, Fossati G, Gritti D, Angoli L, Specchia G. Clinical and angiographic correlates of leukocyte activation in unstable angina. J Am Coll Cardiol 1995;26: 1146-50.
12. Kelley JL, Rozek MM, Suenram CA, Schwartz CJ. Activation of human peripheral blood monocytes by lipoproteins. Am J Pathol 1988;130:223-31.
13. van Oostrom AJ, Rabelink TJ, Verseyden C, Sijmonsma TP, Plokker HW, De Jaegere PP, Castro Cabezas M. Activation of leukocytes by postprandial lipemia in healthy volunteers. Atherosclerosis 2004;177: 175-82.
14. Lehr HA, Krombach F, Münzing S, Bodlaj R, Glaubitt SI, Seiffge D, Hübner C, von Andrian UH, Messmer K. In vitro effects of oxidized low density lipoprotein on CD11b/CD18 and L-selectin presentation on neutrophils and monocytes with relevance for the in vivo situation. Am J Pathol 1995;146:218-27.
15. Zhang WJ, Schwartz E, Wang Y, Attrep J, Li Z, Reaven P. Elevated Concentrations of Nonesterified Fatty Acids Increase Monocyte Expression of CD11b and Adhesion to Endothelial Cells. Arterioscler Thromb Vasc Biol 2006;26:514-9.
16. Alipour A, van Oostrom AJ, Izraeljan A, Verseyden C, Collins JM, Frayn KN, Plokker TW, Elte JW, Castro Cabezas M. Leukocyte Activation by Triglyceride-Rich Lipoproteins. Arterioscler Thromb Vasc Biol 2008;28:792-7.
17. Kansas GS. Selectins and their ligands: current concepts and controversies. Blood 1996;88:3259-87.

18. Weber C. Novel mechanistic concepts for the control of leukocyte transmigration: specialization of integrins, chemokines, and junctional molecules. J Mol Med 2003;81:4-19.
19. Ducker TP, Skubitz KM. Subcellular localization of CD66, CD67, and NCA in human neutrophils. J Leukoc Biol 1992;52:11-16.
20. Kagan A, Dawber TR, Kannel WB, Revotskie N. The Framingham study: a prospective study of coronary heart disease. Fed Proc 1962;21:52–57.
21. The multiple risk factor intervention trial (MRFIT). A national study of primary prevention of coronary heart disease. JAMA 1976;235:825–27.
22. Inoue T, Uchida T, Yaguchi I, Sakai Y, Takayanagi K, Morooka S. Stent-induced expression and activation of the leukocyte integrin Mac-1 is associated with neointimal thickening and restenosis. Circulation 2003;107: 1757–63.