Association of Blood Monocyte and Platelet Markers with Carotid Artery Characteristics: The Atherosclerosis Risk in Communities Carotid MRI Study

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Key Words

Atherosclerosis • Flow cytometry • Magnetic resonance imaging

Abstract

Background: Atherosclerosis is characterized by infiltration of inflammatory cells from circulating blood. Blood cell activation could play an important role in plaque formation. **Methods:** We analyzed the relationship between blood cellular markers and quantitative measures of carotid wall components in 1,546 participants from the ARIC (Atherosclerosis Risk in Communities) Carotid MRI Study. Carotid imaging was performed using a gadolinium contrast-enhanced MRI and cellular phenotyping by flow cytometry. **Results:** Monocyte Toll-like receptor (TLR)-2 is associated with larger plaques, while CD14, myeloperoxidase, and TLR-4 associate with smaller. Platelet CD40L is associated with smaller plaques and thinner caps, while P-selectin is associated with smaller core size. **Conclusions:** Blood cell activation is significantly associated with atherosclerotic changes of the carotid wall.

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Introduction

Atherosclerosis is a chronic, progressive vascular disorder characterized by infiltration of inflammatory cells from circulating blood, including monocyte-derived foam cells and extracellular matrices [1]. Once in the vascular wall, they trigger and propagate inflammatory reactions that are crucial not only in initiation of atherosclerosis, but also in progression of atheromatous plaques and regulation of plaque stability. Despite a wealth of knowledge on the involvement of infiltrating blood cells in atherosclerosis, little is known about the relationship between circulating blood cell activation and carotid artery wall architecture/atherosclerotic plaque characteristics. Circulating leukocytes and platelets are highly reactive to exogenous stimuli such as microbial toxins, notably endotoxins, lipopolysaccharides (LPS), cytokines, and immune mediators. The stimulated cells undergo molecular and cellular changes and secrete active pro-inflammatory molecules that induce endothelial adhesion molecules and facilitate transmigration of blood cells [2–4]. Blood cell activation may therefore play an

important role in contributing to atherogenesis and atheromatous plaque formation. In these exploratory analyses, we postulated that plaque size and composition, measured by a high-resolution magnetic resonance imaging (MRI), are associated with proinflammatory conditions of circulating blood cells. To address this, we analyzed multiple leukocyte and platelet pro-inflammatory molecules in 1,546 participants from the ARIC (Atherosclerosis Risk in Communities) Carotid MRI study using flow cytometry.

To our knowledge, there are no comprehensive studies that have explored the relationships between circulating blood cells and specific parameters of carotid artery wall architecture, as measured by MRI. Therefore, we analyzed the association of multiple cellular pro-inflammatory molecules with carotid artery wall characteristics in the large biracial ARIC Carotid MRI Study cohort.

Methods

Study Design and Participants

The ARIC study is a multicenter, prospective cohort study of atherosclerosis among 15,792 African-American and Caucasian people aged 45–64 years at recruitment (1987–1989) [5]. The ARIC carotid MRI Study was conducted between 2004 and 2006 as a cross-sectional sub-study and was designed to advance our understanding of cellular factors and their association with atheromatous plaques. To increase the prevalence of the informative plaques while maintaining the ability to make population-based inferences, a stratified sampling plan was used. The study sample consisted of 2,066 members of the ARIC study cohort, of which 1,901 underwent a complete MRI examination. Of these 1,901 participants, 1,769 had sufficient-quality MRI scans, with adherence to the MRI protocol. In addition, 73 participants with 1 or more missing values for any of the cellular measurements were excluded, leaving 1,634 subjects in the final analyses. With the multivariate adjusted models, 88 participants had missing data on the adjustment variables, which left a remaining 1,546. The average age of the substudy participants was 71.2 years; 57% were female, 81% white, and 19% African American. The prevalence of risk factors in the ARIC Carotid MRI study participants has been published elsewhere [6]. The present study was approved by the institutional review committees of all participating centers; all participants provided informed consent.

Magnetic Resonance Imaging Protocol

MRI studies were performed on a 1.5T scanner (GE Medical Systems, Milwaukee, Wisc., USA, at three field centers, and Siemens Medical Solutions, Erlangen, Germany, at one field center) equipped with a bilateral 4-element phased array carotid coil (Machnet, The Netherlands). A 3-dimensional time-of-flight magnetic resonance angiogram was acquired through both carotid bifurcations. Detailed black blood MRI images were then acquired through the extracranial carotid bifurcation known to have a thicker maximum wall by the most recent ultrasonograph-

ic study, unless the technologist determined that the contralateral carotid bifurcation wall appeared thicker on the magnetic resonance angiogram. Black blood MRI was achieved using a cardiac-gated, 2-dimensional, double-inversion recovery fast spin-echo sequence, with the inversion time set to suppress the signal of blood. Each participant received an intravenous injection of gadodiamide (Omniscan, GE Amersham, UK), 0.1 mmol/kg body weight, using a power injector. Sixteen transverse T₁-weighted black blood MRI images (acquired resolution, 0.51 × 0.58 × 2.00 mm³; total longitudinal coverage, 3.2 cm) were oriented perpendicular to the vessel and centered at the thickest part of the internal or common carotid artery wall. These 16 slices were acquired 5 min after the injection through the thicker carotid artery using a 2-dimensional double-inversion recovery fast spin-echo sequence, with the inversion time set to suppress the signal of the contrast-enhanced blood pool.

Image Analysis

The MRIs were analyzed by seven experts, blinded to the clinical and laboratory characteristics of the study population. All examinations were assessed for image quality and protocol adherence; examinations that failed to follow these protocols were not analyzed.

Slices were numbered 1 through 16 from proximal to distal; only 8 slices, centered around the slice with the thickest wall, were analyzed. The plaque components were analyzed on the postcontrast black blood MRI series based on the ability of gadolinium enhancement to delineate and enable quantitative size measurements of the fibrous cap [7]; contours were drawn to delineate the outer wall, the lumen, the lipid core, and calcification. The total vessel area included the lumen, the intima, the media, and the adventitia. Wall area was calculated as the difference between total vessel area and lumen area. The derived MRI variable, Normalized Wall Index (NWI), was calculated by dividing the wall area by the total vessel area. Reliability coefficients (R) for the previously mentioned MRI variables were obtained from an internal reliability study [8]. Generally, the reliability for most MRI variables varied from excellent (R > 0.75) to fair to good (0.40 < R < 0.75). However, the limited resolution of the 1.5-T MRI scanner may have contributed to the lower reliability of measurements related to relatively small structural features, such as the fibrous cap thickness. Although reliability based on repeated readings was fair to good for cap thickness measures (R = 0.60), reliability based on repeated scans was poor for cap thickness (R = 0.38). A detailed description of the image analysis methods for the ARIC Carotid MRI Study was published previously [8].

Flow Cytometry

Blood samples were collected in Cyto-Chex[®] BCT vacutainer tubes (Streck, Omaha, Nebr., USA) containing EDTA and a cell membrane stabilizer for blood cells at the field centers [9]. Samples were shipped overnight and analyzed immediately after arrival by flow cytometry [10]. We measured the following markers: (1) platelet CD41 (glycoprotein IIb, GP-IIb), CD61 (GP-IIIa), CD62P (P-selectin) and CD154 (CD40 ligand); (2) monocyte CD14 (LPS receptor), CD162 (P-selectin glycoprotein ligand-1 or PSGL-1), Toll-like receptors 2 (TLR2; CD282) and 4 (TLR4; CD284), and myeloperoxidase (MPO), and (3) platelet-monocyte aggregates (PMA). Platelets were identified and distinguished from non-platelet events (red blood cells and leukocytes) by their

characteristic forward light scatter (FS) versus side scatter (SSC) characteristics (log FS; log SSC) and their fluorescence from FL1 (CD61 and CD41), in the log-log dot plot of FS versus CD61 or CD41. Monocytes were identified by a specific CD14-PC5 staining, and PMAs were analyzed by double staining with anti-CD14 and anti-CD41 and were defined as a population of CD14+ monocytes that are also CD41+. A detailed description of the flow cytometry protocols has been published elsewhere [10]. Data are presented as proportions of cells expressing the antigen (%) and/or the relative levels of antigen expression assessed by the median fluorescent intensity (MFI).

Statistical Analysis

All analyses were based on methods appropriate for a stratified random sampling. In particular, all analyses were weighted by the inverse of the sampling fractions in the 8 sampling strata (4 field centers $\times$ 2 IMT groups). The sampling fractions were based on those persons screened for participation. Those who actually participated were analyzed as a domain for calculating variances and confidence intervals (CIs) of estimators. Analyses were conducted using computer software for descriptive statistics (SAS, version 9.1; SAS Institute Inc., Cary, N.C., USA) or for domain analysis (SUDAAN; RTI International, The Research Triangle, N.C., USA). Finite population correction factors were not applied. Tests of differences in weighted means or proportions between groups were from weighted linear or logistic regression models that accounted for the sampling. Adjusted means and proportions by subgroup of interest were calculated using the SUDAAN REGRESSION procedure for continuous variables and the LOGISTIC procedure for dichotomous variables, with predicted values calculated as sample means of the adjusting variables. Wall thickness and wall volume were analyzed in the full data set of 1,546 participants. Because of the resolution constraints of the MRI scan, we restricted consideration of lipid core to the 1,064 participants whose maximum wall thickness was ≥ 1.5 mm (weighted percentage, 62%). Only four lipid cores were excluded using this threshold. Measures of lipid core volume and area and fibrous cap thickness were analyzed as continuous variables among those 513 participants with a lipid core. An additional analysis considered correlates of lipid core presence as a dichotomous variable. Standardized regression coefficients are presented for linear and logistic regression models, standardizing by one SD of exposure and outcome (for continuous outcomes), with adjustment for age, race, and sex.

Results

Association of Cellular Markers and Aggregates with Carotid Wall Volume

We investigated the relationships of blood platelet and leukocyte markers and cellular aggregates with continuous carotid wall thickness or volume measures (table 1).

Monocyte TLR-2 was positively associated with total wall volume, while monocyte CD14 (MFI), TLR-4 (% and MFI), MPO (MFI), and platelet CD40L (MFI) were negatively associated with total wall volume. When the analy-

Table 1. ARIC Carotid MRI Study: relationship between continuous wall thickness measures and flow cytometry measures

Flow cytometry variables	Total wall volume (n = 1,546)	Maximum segmental wall thickness (n = 1,546)
Platelets		
GPIIb, MFI	0.06 (0.19)	-0.03 (0.48)
GPIIIa, MFI	-0.03 (0.52)	-0.01 (0.84)
P-selectin, %	0.02 (0.60)	0.01 (0.74)
P-selectin, MFI	0.003 (0.95)	-0.03 (0.36)
CD40L, %	0.07 (0.09)	0.04 (0.25)
CD40L, MFI	-0.1 (0.03)	-0.04 (0.17)
Monocytes		
CD14, MFI	-0.14 (0.004)	-0.02 (0.62)
TLR-2, %	0.11 (0.03)	0.07 (0.04)
TLR-2, MFI	0.09 (0.06)	0.06 (0.12)
TLR-4, %	-0.12 (0.02)	-0.01 (0.86)
TLR-4, MFI	-0.13 (0.007)	-0.002 (0.95)
PSGL-1, MFI	0.07 (0.15)	0.01 (0.80)
MPO, MFI	-0.10 (0.03)	-0.05 (0.15)
Cellular aggregates		
PMA, %	0.06 (0.26)	0.02 (0.59)

Results are adjusted for age, race, gender, LDL-C, HDL-C, TG, CRP, BMI, waist/hip, diabetes, smoking, lipid-lowering therapy, and antihypertensive medications.

Standardized beta coefficients, p value in parentheses. Number of SD differences in MRI variables associated with 1 SD increment in flow cytometry measures.

sis was restricted to maximum segmental wall thickness, only monocyte TLR-2 remained positively associated.

Association of flow cytometry markers (weighted mean values) with wall thickness was further analyzed by comparing the markers in participants with maximal wall thickness <1.5 vs. ≥ 1.5 mm. Subjects with maximal wall thickness ≥ 1.5 mm had statistically significantly higher frequencies of platelets expressing CD40L (3 ± 2.7 vs. 2.6 ± 2 , $p < 0.01$), and lower monocyte intracellular MPO levels (89.6 ± 24.5 vs. 96.6 ± 25.7 , $p < 0.001$). There was no difference in the mean levels of other flow cytometry markers between participants with wall thickness ≥ 1.5 vs. <1.5 mm.

Association of Cellular Markers and Aggregates with Fibrous Cap Thickness

Analysis of the relationship between fibrous cap measures and flow cytometry measures was restricted to participants with maximal wall thickness ≥ 1.5 mm and

Table 2. ARIC Carotid MRI Study: flow cytometry profile of fibrous cap measures and flow cytometry measures, restricted to participants with max wall thickness ≥ 1.5 mm and lipid core present

Flow cytometry variable	Mean cap thickness (n = 499)	Mean minimum cap thickness (n = 501)
Platelets		
GP IIb, MFI	-0.02 (0.24)	-0.003 (0.81)
GP IIIa, MFI	-0.03 (0.15)	0.01 (0.69)
P-selectin, %	0.01 (0.67)	0.01 (0.67)
P-selectin, MFI	0.01 (0.60)	0.004 (0.80)
CD40L, %	-0.01 (0.73)	-0.00 (0.99)
CD40L, MFI	-0.03 (0.04)	-0.02 (0.05)
Leukocytes		
CD14, MFI	-0.003 (0.85)	-0.01 (0.39)
TLR-2, %	0.02 (0.18)	0.01 (0.45)
TLR-2, MFI	0.01 (0.29)	-0.002 (0.85)
TLR-4, %	0.03 (0.12)	0.01 (0.26)
TLR-4, MFI	0.001 (0.94)	0.001 (0.96)
PSGL-1, MFI	0.02 (0.30)	0.003 (0.85)
MPO, MFI	0.01 (0.55)	-0.003 (0.86)
Cellular aggregates		
PMA, %	0.03 (0.03)	0.02 (0.13)

Results are adjusted for age, race and gender, LDL-C, HDL-C, TG, CRP, BMI, waist/hip, diabetes, smoking, lipid-lowering therapy, antihypertensive medications (fibrous cap also adjusted for wall thickness).

Standardized beta coefficients, p value in parentheses. Number of SD differences in MRI variables associated with 1 SD increment in flow cytometry measures.

Table 3. ARIC Carotid MRI Study: flow cytometry profile for continuous lipid core measures restricted to participants with max wall thickness >1.5 mm

Flow cytometry variable	Total lipid core volume (n = 513)	Maximum lipid core area (n = 513)
Platelets		
GP IIb, MFI	-0.01 (0.74)	-0.002 (0.62)
GP IIIa, MFI	0.02 (0.65)	-0.001 (0.90)
P-selectin, %	-0.04 (0.18)	-0.01 (0.21)
P-selectin, MFI	-0.08 (0.03)	-0.01 (0.05)
CD40L, %	0.01 (0.72)	0.004 (0.30)
CD40L, MFI	0.03 (0.25)	0.003 (0.32)
Monocytes		
CD14, MFI	0.06 (0.10)	0.01 (0.06)
TLR-2, %	-0.00 (0.99)	0.003 (0.53)
TLR-2, MFI	-0.02 (0.68)	0.002 (0.74)
TLR-4, %	-0.01 (0.75)	-0.00 (0.97)
TLR-4, MFI	0.04 (0.22)	0.004 (0.44)
PSGL-1, MFI	-0.01 (0.83)	-0.001 (0.77)
MPO, MFI	-0.01 (0.66)	0.001 (0.83)
Cellular aggregates		
PMA, %	-0.03 (0.35)	-0.01 (0.15)

Results are adjusted for age, race and gender, LDL-C, HDL-C, TG, CRP, BMI, waist/hip, diabetes, smoking, lipid-lowering therapy, antihypertensive medications (lipid core also adjusted for total wall volume).

Standardized beta coefficients, p value in parentheses. Number of SD differences in MRI variables associated with 1 SD increment in flow cytometry measures.

presence of a lipid core. After adjustments for age, race, sex, and conventional cardiovascular risk factors, only platelet CD40L levels were inversely associated with mean cap thickness and mean minimum cap thickness, while platelet-monocyte aggregates (%) were associated with greater mean cap thickness (table 2).

Association of Cellular Markers and Aggregates with Lipid Core Size

Table 3 shows the relationship between lipid core and flow cytometry measures, restricted to participants with maximum wall thickness >1.5 mm. Only the platelet level of P-selectin was statistically significantly associated (inversely) with total lipid core volume, and the significance was borderline with maximum lipid core area. Participants who had lipid core had statistically significantly lower platelet GPIIb levels (77.5 ± 12.9 vs. 79.7 ± 11.5 , $p = 0.02$) and monocyte PSGL-1 levels (110.8 ± 15.3 vs. 113.7 ± 15.5 , $p = 0.01$) than those without a detectable lipid core.

Discussion

In this cross-sectional, population-based study, we examined the association of circulating blood cellular markers with atherosclerotic plaque size and composition as measured by MRI. For the thickest area of the carotid wall, we assessed plaque size, expressed as total wall thickness and volume, and plaque composition, assessed as lipid core quantity and fibrous cap thickness. We found that several pro-inflammatory markers of circulating blood monocytes and platelets were associated with plaque size and composition. Notably, monocyte TLR-2 was associated with greater plaque size, heterotypic cell aggregates were associated with greater fibrous cap thickness, monocyte MPO and TLR-4 were associated with smaller plaque size, and platelet activation markers were inversely associated with both plaque size and the size of plaque components. There was also a weak inverse association between platelet GPIIb, monocyte PSGL-1, and

plaque lipid core. These results suggest the involvement of different pro-inflammatory cellular markers with plaque formation, fibrous deposition, and tissue necrosis. It may be speculated that blood cell inflammatory molecules may impact endothelium, thereby altering transmigration of monocytes. Furthermore, once entering the vascular wall, different monocyte inflammatory molecules may contribute to plaque formation, plaque core necrosis/apoptosis, and cap matrix deposition.

Activation of monocytes and macrophages is an important step in many inflammatory diseases including atherosclerosis. Monocytes recruited through the activated endothelium differentiate into macrophages, which take in lipids to become foam cells, an early step in atherogenesis. Several molecules can ligate TLRs on these cells leading to inflammation and tissue damage. TLR-2 and TLR-4 play key roles in initiating inflammatory responses, and their upregulation in atherosclerotic lesions has been documented in animal models and in humans [11–13]. Upon activation, TLRs use signaling pathways leading to the production of pro-inflammatory cytokines and chemokines. The receptor complex of CD14 and TLR-4 in peripheral monocytes appears to be the principal receptor complex of LPS, mediating activation of transcription factor NF- κ B, which plays an important role in inducing synthesis of proinflammatory cytokines [14–16].

There are limited data available regarding the association of circulating, blood cells expressing TLRs with plaque size and composition. In peripheral blood, monocytes represent the predominant site of TLR expression. Several studies reported monocyte TLR upregulation in relation to coronary artery disease [17] or heart failure after acute myocardial infarction [18]. Increased TLR-2 and TLR-4 expression was also reported in monocytes from patients with type-1 diabetes, indicating their contribution to a proinflammatory state of this disease [19]. In our study, monocyte TLR-4 expression was independently, significantly and inversely associated with wall thickness. It is possible that lower TLR-4 levels indicate downregulation of TLR-4 due to its internalization, a process that was documented in mouse peritoneal macrophages [20]. In atherosclerotic ApoE^{-/-} mice, increased expression over time of TLR-2 and -4 in atherosclerotic plaques and on blood monocytes correlated with the state of atherosclerotic disease. Our results, showing an independent association of the monocyte TLRs with atherosclerotic wall thickness, corroborate these animal studies in a large human study.

CD14 (a monocyte marker) levels on the surface of circulating monocytes and intracellular levels of MPO were inversely associated with total wall volume and may be explained by the depletion of MPO during monocyte activation when MPO is released into plasma [21]. This possible explanation is supported by reports that plasma MPO levels are increased in coronary heart diseases and are associated with increased risk of major atherosclerotic coronary events [22, 23]. This notion is further strengthened by reports that humans with MPO deficiency, or an MPO polymorphism associated with reduced MPO expression, have a reduced risk of cardiovascular disease [24, 25]. Brevetti et al. [26] reported a reduced neutrophil MPO content in coronary artery disease patients with peripheral artery disease (PAD), and Monaco et al. [27] reported that neutrophils were activated and depleted of MPO in unstable angina patients.

Our analysis of platelet markers indicated that platelet activation is associated with smaller plaque size, smaller core size, and thinner caps. Notably, participants with increased total wall volume, or with increased mean cap thickness, had significantly lower expression of platelet CD40L. Participants with higher total lipid core volume and bigger maximum lipid core area had lower expression of P-selectin. These findings suggest ongoing platelet activation, with P-selectin and CD40L likely being released into circulation, where they are engaged in an array of proatherogenic reactions.

Platelets are known to contribute to the progression of atherosclerosis [28]. P-selectin mediates leukocyte transmigration into the vessel wall and interacts with PSGL-1 to activate leukocytes and form platelet-leukocyte aggregates [29, 31]. Recently, it has been shown that activated platelet P-selectin and platelet-monocyte aggregates contribute to progression of atherosclerosis in ApoE^{-/-} mice [32].

Despite a number of studies related to platelet activation, there are few human studies of correlations between platelet activation markers and plaque size/composition. In contrast to our study, Koyama et al. reported a positive association of platelet P-selectin with carotid artery atherosclerotic wall thickness (intima-media thickness) in 517 subjects [33], while another study showed independent association of platelet CD63 but not P-selectin in 105 patients with diabetes mellitus type 2 [34]. These studies differ in how they measure wall thickness and the cut-off values used, as well as in study design and patient characteristics. It is possible that lower platelet CD40L and P-selectin expression in our study may be attributed to engagement of the activation markers to platelet-platelet

and platelet-leukocyte aggregate formation. Platelet-leukocyte aggregates are considered to be a more sensitive marker of platelet activation than P-selectin because of their longer duration in blood circulation [35]. Heterotypic circulating cellular aggregates between platelets and leukocytes have been reported in other studies in relation to various diseases and conditions, and they are considered to propagate the inflammatory process of atherosclerosis [36, 37]. In our study, platelet-monocyte aggregates were positively associated with plaque cap thickness.

There are several limitations to the current study. First, the cross-sectional nature of our study does not allow us to establish temporality in the associations. Second, even though this is supported by literature, only a single plaque was characterized, and we are generalizing a single plaque to be representative of the entire cardiovascular system. Furthermore, MRI measurement reliability depends on structure size, so cap thickness measurements were not as reliable as wall thickness or core size. Finally, multiple statistical associations were examined, and some of those at nominal significance ($p < 0.05$) were likely chance associations. Thus, based on biologic and mechanistic plausibility, our results suggest a pathogenic role of expressed cellular markers, but may underestimate the strength of the associations and do not establish the antecedent role of the markers.

There are also strengths to the current study. First, the study group was selected from the well-characterized, biracial population-based ARIC cohort. To our knowledge, this is the largest population-based study investigating the relationship of blood cellular markers with quantita-

tive measures of carotid wall components using a gadolinium contrast-enhanced MRI examination. Second, a standardized MRI protocol with a central reading facility was used; extensive quality control data were collected, allowing assessment of the MRI measures and the expression of blood platelet and monocyte markers. Finally, the present study has technical advantages over previous studies, since flow cytometry allows for measuring MPO levels in individual cells, independent of the total white blood cell count, which is a major factor affecting measurements of serum and plasma MPO levels.

In summary, our data indicate that both monocyte and platelet activation are associated with distinct carotid arterial wall characteristics. Additional studies are needed to investigate the relationship between blood cellular markers and atherosclerosis burden.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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