Published in final edited form as:

Int J Cardiol. 2015 February 15; 181: 57–64. doi:10.1016/j.ijcard.2014.11.156.

Osteogenic Monocytes within the Coronary Circulation and their Association with Plaque Vulnerability in Patients with Early Atherosclerosis

Julia Collin, MD¹, Mario Gössl, MD², Yoshiki Matsuo, MD, PhD³, Rebecca R. Cilluffo, MD⁴, Andreas J. Flammer, MD⁵, Darrell Loeffler⁴, Ryan J. Lennon, MSc⁶, Robert D. Simari, MD, Daniel B. Spoon, MD⁴, Raimund Erbel, MD¹, Lilach O. Lerman, MD, PhD^{3,8}, Sundeep Khosla, MD⁷, and Amir Lerman, MD⁴

¹Department of Cardiology, West-German Heart-Center - University Hospital Essen, Essen, GERMANY

²Department of Cardiology, Dean Clinic – Dean St. Mary's Outpatient Hospital Center, Madison, Wisconsin, USA

³Department of Cardiovascular Medicine, Wakayama Medical University, Wakayama, JAPAN

⁴Division of Cardiovascular Diseases and Internal Medicine, Mayo Clinic and College of Medicine, Rochester, Minnesota, USA

⁵Department of Cardiology, University Hospital of Zurich, Zurich, SWITZERLAND

⁶Division of Biomedical Statistics and Informatics, Mayo Clinic and College of Medicine, Rochester, Minnesota, USA

⁷Endocrinology, Mayo Clinic and College of Medicine, Rochester, Minnesota, USA

⁸Division of Nephrology and Hypertension, Mayo Clinic and College of Medicine, Rochester, Minnesota, USA

Abstract

Objectives—This study tests the hypothesis that circulating mononuclear cells expressing osteocalcin (OCN) and bone alkaline phosphatase (BAP) are associated with distinct plaque tissue components in patients with early coronary atherosclerosis.

Background—Plaque characteristics implying vulnerability develop at the earliest stage of coronary atherosclerosis. Increasing evidence indicates that cells from the myeloid lineage might serve as important mediators of destabilization. Plaque burden and its components were assessed

© 2014 Elsevier Ireland Ltd. All rights reserved.

Address for correspondence: Dr. Amir Lerman, Division of Cardiovascular Diseases, Mayo Clinic Rochester, 200 First Street SW, Rochester, MN 55905, USA., lerman.amir@mayo.edu. Tel: +1 507 255 4152 Fax: +1 507 255 2550.

All authors have no relationships relevant to the contents of this paper to disclose.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

regarding their relationship to monocytes carrying both pro-inflammatory (CD14) and osteogenic surface markers OCN and BAP.

Methods—Twenty-three patients with angiographically non-obstructive coronary artery disease underwent coronary endothelial function assessment and virtual histology-intravascular ultrasound of the left coronary artery. Plaque composition was characterized in the total segment (TS) and in the target lesion (TL) containing the highest amount of plaque burden. Blood samples were collected simultaneously from the aorta and the coronary sinus. Circulating cell counts were then identified from each sample and a gradient across the coronary circulation was determined.

Results—Circulating CD14+/BAP+/OCN+ monocytes correlate with the extent of necrotic core and calcification ($r=0.53$, $p=0.010$; $r=0.55$, $p=0.006$, respectively). Importantly, coronary retention of CD14+/OCN+ cells also correlate with the amount of necrotic core and calcification ($r=0.61$, $p=0.003$; $r=0.61$, $p=0.003$) respectively.

Conclusions—Our study links CD14+/BAP+/OCN+ monocytes to the pathologic remodeling of the coronary circulation and therefore associates these cells with plaque destabilization in patients with early coronary atherosclerosis.

Keywords

Imaging; atherosclerosis; blood cells; vulnerable plaque

1. Introduction

Atherosclerosis is well known as a chronic inflammatory process. Pro-inflammatory monocytes are thought to contribute significantly to atherosclerosis onset and progression [1]. CD14 expression, among others, is associated with pro-inflammatory activation of monocytes and plays an important role in the interaction between leukocytes and the endothelium[2]. Since CD14+ monocytes likely play an important role in atherosclerosis by maintaining a chronic inflammatory response, they may also be involved in the pathologic remodeling of the plaque structure itself. In a recent study, myeloid calcifying cells from the monocyte/macrophage lineage with co-expression of osteocalcin (OCN) and bone alkaline phosphatase (BAP) showed pro-calcific activity *in vitro* and *in vivo* and were found to be abundant in carotid atherosclerotic plaques in patients with type 2 diabetes[3]. It can be speculated that these inflammatory cells featuring osteogenic properties also influence coronary intra-plaque architecture.

The expansion of the greyscale intravascular ultrasound (IVUS) featuring spectral analysis of the radiofrequency dataset shows the potential to distinguish certain tissue components in the lesion using virtual histology (VH)[4]. The accuracy of this tool for histologic characterization of atherosclerotic plaques was demonstrated in *in vivo* studies of coronary [5] and carotid plaques[6]. It has been previously demonstrated that coronary artery segments with endothelial dysfunction (ED) are associated with distinct plaque characteristics implying plaque vulnerability [7] already in the very early stage of atherosclerosis. Due to the power of visualizing already early plaque changes, in the current study, VH-IVUS was used to examine whether plaque instability involves osteogenic monocytes.

Thus, we tested the hypothesis, that osteogenic monocytes are correlated with particular plaque components determined by virtual histology-intravascular ultrasound (VH-IVUS) and are retained in the coronary circulation in patients with early atherosclerosis. Therefore, we assessed the histological characteristics of each examined vessel and focused on the segment with the highest plaque burden to address the possible relationship between osteogenic monocytes and a particular plaque texture.

2. Methods and Materials

2.1 Study subjects

The study was approved by the Institutional Review Board of Mayo Clinic and complies with the Declaration of Helsinki. All subjects provided written, informed consent. Patients were enrolled between February 2011 and July 2012 and included 23 subjects who underwent coronary angiography, coronary endothelial function testing, greyscale and VH-IVUS assessment.

We included male and female subjects between age 18 and 85. Each was referred by their referring cardiologist to the cardiac catheterization lab for coronary angiography. The procedure included standard clinically indicated endothelial function testing using acetylcholine. Patients without significant structural coronary artery disease (stenosis less than 30% in any coronary segment), but detected ED were included. These patients were presumed to have early coronary atherosclerosis [8, 9].

Exclusion criteria for the present study were heart failure with an ejection fraction less than 50%, unstable angina, and myocardial infarction or angioplasty within 6 month prior to entry into the study. Patients were excluded with luminal diameter of the study vessel less than 2.5 mm, severe tortuosity of the study vessel, or any other relevant anatomical reasons that the investigator deemed the patient to be inappropriate for the study.

2.2 Coronary angiography and invasive endothelial function testing

Patients underwent a diagnostic coronary angiography using standard clinical protocols [10, 11]. Simultaneous blood was drawn from the left coronary artery (30 ml) and the coronary sinus catheter (30 ml) for flow cytometry analyses. All subjects underwent assessment of endothelium-dependent coronary vasoreactivity, as previously described [8, 10–12].

Microvascular ED was defined as an increase in CBF of <50% and epicardial ED as a decrease in epicardial coronary artery diameter of more than 20% in response to the maximal dose of acetylcholine (10^{-4} M). Endothelium-independent microvascular function was determined by the coronary flow reserve, which is the ratio of the APV at maximal hyperemia (induced by intracoronary adenosine (24–60 μ g)) to the APV at baseline. Patients with either microvascular ED or epicardial ED were considered to have early atherosclerosis.

2.3 Flow cytometry

Fresh whole blood samples were processed for isolation of peripheral blood mononuclear cells by Ficoll density gradient. Immunofluorescent cell staining was performed by using

CD14 Per CP-Cy5.5 (Beckton-Dickinson), alkaline phosphatase-allophycocyanin (R&D Systems), and the appropriate isotype controls [Iso CD14 Per CP-CY5.5 (Beckton-Dickinson 550927); Iso AP APC (R&D Systems IC002B)].

OCN staining was performed accordingly to previous protocols [13–16] by using anti-human OCN antibody (Santa Cruz Biotechnology) and a fluorescein isothiocyanate secondary antibody [Jackson ImmunoResearch; isotype: Iso OCN PE (R&D Systems IC002P)]. As previously demonstrated OCN surface co-staining is feasible and reliable although OCN is a secreted protein. Therefore no permeabilization was required [14].

A total of 150,000 events within the monocyte gate were analyzed by using CellQuest software (Beckton-Dickinson), as previously described [17]. Subsequent analysis following flow cytometry was performed in a blinded manner. Threshold settings for background of corresponding isotype controls included 0.3% of positive gated cells, except for OCN, where 1.0% background was used (Figure 1).

2.4 Determination of the net gradient

The trans-cardiac gradient was determined by subtracting the coronary sinus cell number from the aortic cell number. The cell net gradient was calculated by multiplying trans-cardiac gradient with coronary blood flow as determined by intracoronary Doppler [10, 18]. A positive net gradient indicated retention, whereas a negative gradient indicated release of osteogenic cells from the coronary circulation.

2.5 IVUS acquisition and VH-IVUS image analyses

Following angiography, greyscale and VH-IVUS were performed using phased-array 20 MHz IVUS catheters and the S5 Imaging System (Eagle-Eye Gold, Volcano Corporation, Rancho Cordova, California) in the left anterior descending artery (LAD), [8, 12, 19–21]. The image examination was performed by a blinded observer in the IVUS Imaging center at Mayo Clinic, Rochester. Luminal and medial-adventitial borders were manually adjusted for each analyzable cross-sectional frame from the entire pullback length [7, 8, 12, 19, 22].

2.6 Definition of segments

Total segment (TS)—After adjusting borders in each cross-sectional area (1-mm intervals) for the entire analyzed segment, volume of plaque burden and tissue components were calculated and subsequently normalized for segment length resulting in average area (mm^2).

Target lesion (TL)—Ten millimeters of the TL section were chosen from the segment with the highest plaque burden and normalized for length [23] (Figure 2a, b, c).

2.7 Statistical analysis

Data are shown as median (and 25th, 75th percentile range) for non-normally distributed variables. Otherwise mean $\pm$ standard deviation was used. Cell numbers were logarithmically regressed and adjusted for age to enable comparison with the control group.

Pearson's chi-squared test for nominal variables was used for comparison of patient characteristics and medication. Comparison of cell numbers between study patients and control subjects was achieved using Wilcoxon signed-rank test.

All correlations were done using Spearman coefficient. Adjustment for multiple comparisons required data transformation to their rank values (non-parametric approach). For each imaging variable, a multiple regression model was fit with the monocyte variables as the set of independent variables. The F-test for the model worked as an overall test for the imaging variable association with the monocyte variables. We used a 0.005 significance level as the first screening level. For row values <0.005 , individual p-values within that row were considered significant <0.01 (Table 3). Otherwise, p-values <0.05 were considered statistically significant. JMP software 9.0.1 (SAS Institute, Cary, NC) was used for statistical analysis.

3. Results

3.1 Patient characteristics

Clinical data and blood samples from 23 patients were analyzed. Relevant clinical data is shown in Table 1. No sex-based differences were detected due to the small sample size.

Endothelial functional testing was assessed and epicardial ED was detected in all study patients with a median of -16.2 [-25.7 , -6.0] % change in coronary blood flow. Additionally, nineteen patients (83%) had an endothelium-independent microvascular ED (21.9 [-28.3 , 57.0] % change in coronary artery diameter). IVUS segment analysis data and additional virtual characteristics are presented in (Table 2). The distribution of plaque burden in the 23 total segments and target lesions is shown in (Figure 3).

3.2 Correlation of net gradient to coronary imaging parameters

The calculated net gradient of CD14+/OCN+ monocytes in the study group showed a median of $-1,644$ (25. quartile: $-22,136$; $2,062$) cells/ml/min. After adjusting for multiple comparisons, the net gradient of CD14+/OCN+ cells correlated with necrotic core (NC) and calcification in the TL ($r=0.61$, $p=0.003$ (Figure 4) and $r=0.61$, $p=0.003$) respectively. There was no correlation with simple CD14+ cells alone. The correlations with plaque burden or other tissue components and the net gradient of CD14+/OCN+ monocytes were not found to be statistically significant (Table 3). No significant correlation with the net gradient of CD14+/OCN+ and additional BAP expression and any tissue component has been found (for NC in the TL: $r=0.32$, $p=0.14$).

Collectively, the majority of the study population had release of CD14+/OCN+ monocytes and this is associated with lower extent of calcification and NC in the target lesion. The presented correlations are all referred to the average area of the total lesion/target lesion to account for the different segment length. The volumetric data showed a similar correlation and therefore is not shown separately.

3.3 Correlation of circulating osteogenic CD14+ monocytes with vessel wall features

The median number of circulating CD14+/BAP+/OCN+ monocytes was 51 (12, 158) cells/100,000 cells. The NC in the TL showed significant correlation to the circulating CD14+/BAP+/OCN+ cells ($r=0.53$, $p=0.01$) (Figure 5). Additional correlation was found with calcification ($r=0.55$, $p=0.006$) in this segment. Only the expression of osteogenic surface markers showed a correlation to NC and calcification (Table 3). The correlation between plaque burden or other tissue components and osteogenic monocytes was not found to be statistically significant. Furthermore, the expression of CD14 alone had no impact on any correlation to the plaque burden or any plaque component.

A decreased extent of calcification in the target lesion ($< 0.04 \text{ mm}^2$) was associated with lower numbers of circulating CD14+/OCN+ and CD14+/OCN+/BAP+ monocytes compared to patients with more calcification ($> 0.04 \text{ mm}^2$) in the study group ($p=0.0138$ and $p=0.0288$, respectively). In a similar manner, minor extent of NC ($< 0.12 \text{ mm}^2$) showed a lower number of circulating CD14+/OCN+ and CD14+/OCN+/BAP+ monocytes compared to higher NC burden ($> 0.12 \text{ mm}^2$; $p=0.0116$ and 0.0266), respectively.

Taken together, the total number of circulating CD14+/OCN+ and CD14+/OCN+/BAP+ cells is associated with a higher amount of NC and calcification in the target lesion.

4. Discussion

4.1 Osteogenic monocytes and plaque properties

The current study demonstrates that there is an association between osteogenic monocytes in the coronary microcirculation and specific plaque properties of patients with early coronary atherosclerosis. The release of osteogenic monocytes from the coronary circulation correlates significantly with lower degree of calcification and NC, whereas retention of these same cells is associated with an increase in plaque components with a more vulnerable plaque composition.

The fluctuation of endothelial progenitor cells through the coronary circulation has been investigated [10]. The current study focuses on cells from the monocytes/macrophage lineage which are known to be highly involved in the process of plaque development [24] and correlated them to coronary imaging. Fadini already showed the calcifying potential for OCN+ myeloid cells in mice [3] and macrophages expressing OCN were found to accumulate in the border region of the NC in human carotid atherosclerotic plaques [25]. Therefore, it seems reasonable that myeloid cells exhibiting osteogenic features are also involved in the mechanism of coronary plaque development. In the context of the association of necrotic core extent and segmental endothelial dysfunction [7], a relationship between tissue characteristics exemplifying plaque vulnerability and osteogenic monocytes in the very early stage of coronary atherosclerosis was detected.

Having in mind that atherosclerosis is a generalized disease and affects the entire vascular bed, this study focuses and emphasizes dynamic changes in the coronary circulation through the trans-cardiac gradient.

4.2 Release of osteogenic monocytes from the coronary circulation

In contrast to Gossel et al. previous study where coronary retention of osteogenic endothelial progenitor cells was observed in patients with endothelial dysfunction [10], CD14+/OCN+ monocytes tend rather to be released than to be retained in the coronary circulation. The release of CD14+/OCN+ cells was associated with a lower extent of NC and calcification and therefore might share protective properties. Monocyte efflux out of lesions was already shown to be associated with disease regression [26]. Zooming into the coronary circulation the vascular wall may serve as a potential source for multipotent cells. Recent findings identified the postnatal murine aorta as a reservoir for multipotent hematopoietic stem and progenitor cells that includes already lineage-directed monocyte and macrophage precursors [27]. Multipotent vascular stem cells were found in the vascular wall and therefore support the hypothesis of de-novo differentiation into various cells [28, 29].

The release of osteogenic monocytes might share a protective impact on plaque formation, whereas an aberrant activation and differentiation might lead the cells towards the coronary plaque. Aberrant activation has been shown and moreover the following impact in onset and progression of vascular disease [29, 30].

Previous studies already reported a permanent interchange of hematopoietic stem and progenitor cells between the circulation and extra-medullary tissues, resulting in a migratory pool, located also in the vasculature, that is capable of differentiating into mature myeloid cells [31]. Therefore, the vessel wall might illustrate an additional source for cell recruitment also in humans [32].

In contrary to our findings, recent studies suggest only a minor amount of tissue-resident macrophages are derived from the bone marrow [33, 34]. Heidt et al. demonstrated the proliferative potential of cardiac-resident macrophages in the murine heart independent of circulating monocytes in the steady state [33]. All in all, cells from the monocytes/macrophages lineage seem to consist of heterogenic subpopulations [35]. But still, less is known about the full transferability in humans since most studies are dealing with animal models. Although the contribution of circulating monocytes to tissue macrophages is small, these observations are mostly referred to a steady state. Several diseases are able to disturb this sensitive balance and have the ability to recruit cells from the circulation. After acute myocardial ischemia, resident macrophages vanish due to apoptosis and emigration but the self-renewal capacity starts not before the initial replacement of resident macrophages by circulating monocytes. We also observed an interaction of circulating cells and atherosclerotic lesions, although release of cells was leading. Since models for different stages of atherosclerosis are not totally understood so far, further investigation of cellular dynamics is necessary.

4.3 Relationship between net gradient and circulating cells

The shift from release to retention of cells within the coronary circulation in patients with increased NC burden and calcification might be caused by an I) increasingly depleted reservoir of local resident stem and progenitor cells in the vascular bed and/or a II) shift of de-novo differentiated vascular cells into the plaque. The persistent chronic inflammatory

response may cause bone marrow exhaustion and therefore decrease cell mobilization as already shown for endothelial progenitor cells [36] but seems to be unlikely since we analyzed coronary plaques from an early age. The aberrant activation and thus cells undergoing abnormal differentiation rather might be considered. A potential result of aberrant differentiated cells is the promotion of vascular disease and might be the underlying reason for the complexity of cell phenotypes in atherosclerotic plaques [28, 30, 37].

A possible explanation for the increased circulating cell numbers is that the inflammatory lesion may trigger monocyte mobilization from the bone marrow causing the release of cells from the vasculature decreases and results in a time shifted peak for osteogenic monocytes. To analyze the coronary dynamics we calculated the net gradient as a useful tool so that coronary segments regarding their cell capacity could be comparable. For the determination of the net gradient we analyzed samples from the LAS and the coronary sinus. The main reason for analyzing the LAD is the ability to assess the gradient across this circulation by obtaining simultaneous sampling from the coronary sinus, which drains the LAD distribution. For this reason the IVUS-analysis was also performed on these coronary segments.

4.4 Retention of osteogenic monocytes within the coronary circulation

A positive net gradient of osteogenic monocytes implies that there is retention of these cells which are associated with a larger extent of NC and calcification, which reflects a plaque composition with vulnerable plaque characteristics [7]. The recruitment and extravasation of circulating monocytes into the plaque is a critical step in terms of disease progression [38, 39]. Subsequent maturation of the monocytes into macrophages within the early atherosclerotic plaque causes an unbalanced turnover of cell from the monocytes/macrophage line within the plaque due to progressive influx and proliferation of macrophages. Apoptosis with decreasing phagocytic clearance and migration into the lesion leads to debris congregation and promotes lesion expansion by triggering inadequate inflammatory response [40, 41].

4.5 Osteogenic monocytes and plaque composition

Circulating cells reach the target vessel within the blood stream and then migrate through the vessel wall. Three possibilities are suggested for osteogenic cells to participate in the pro-calcific process. First, by differentiating into the mesenchymal lineage and promoting active calcification [42]; second, by secreting pro-calcific cytokines and therefore stimulates other cells to calcify [12, 43]; and third, by undergoing apoptosis, which leads to calcification by itself [44]. Since there was no correlation to the plaque size, but to NC and calcification extent, the osteogenic subset seems to be rather influence the plaque composition towards a more vulnerable phenotype than plaque enlargement. The procalcific properties of osteogenic monocytes were recently shown in patients with type 2 diabetes [3] and might explain the correlation to the assessed calcification. The additional correlation to NC extent may be caused by apoptosis of myeloid cells after incorporation [45].

Comparing the total segment analysis and the target lesion, both dispose of mostly fibrous followed, in descending order, by necrotic core, calcification and fibro-fatty. Whereas the

plaque structure is in both analyses mostly the same, by definition the amount of plaque burden differs in favor of the target lesion. The obtained results for amount of total plaque burden and components we see shows similar levels as previously described for early atherosclerosis [7]. Therefore we assume for these lesions with comparable composition of calcification and necrotic core amount the equal characteristics of vulnerable plaques.

The present findings are important for advancing further understanding of dynamics in atherosclerotic lesions, since plaque burden and composition affect cardiac outcome markedly. The lack of coronary plaque burden assessed by cardiac computed tomography is associated with a period of 2 years free of major adverse cardiac events (MACE) as seen in the ROMICAT trial [46]. In terms of plaque dynamics and influence on drug treatment D'Ascenzo et al. performed a meta-analysis showing that pharmaceutical-induced plaque regression significantly reduces myocardial infarction and revascularization, however MACE were not reduced [47]. Concisely, the presence of coronary atherosclerotic plaques and their dynamics have an impact on future cardiac events. Therefore, we investigated changes in the coronary microcirculation on the cellular level in order to get more insights of the underlying mechanisms of plaque regulation.

We showed an association between circulating osteogenic monocytes and plaque destabilization already in the very early development. Thus, we can contribute another small component in the complex pathways of atherogenesis, emphasizing that manipulating plaque growth could be a major target in the causal treatment of atherosclerosis.

4.6 Limitations of the study

The length of the total analyzed segments was highly variable and may have introduced inaccuracy in the calculated average of longer segments. Second, LAD calcification are abundant more in the proximal 60 mm (where the TL was always located) underscoring the notion that coronary artery diseases as early as coronary endothelial dysfunction have a segmental pattern [7, 48].

Our results are limited by the lacking capability to clarify the definite disposition of cells or the mechanisms leading to release or retention. Besides these limitations, the association of these results with longitudinal follow-up data in future studies would be an interesting prospect to investigate plaque development and intra-structural changes over time. Additionally, in the present setting we did not include patients with more severe and advanced atherosclerotic lesions. To investigate the association with osteogenic monocytes in these patients would expand our present findings.

New strategies of targeting already at primary cell interaction through integrins, e.g. or using nanoparticles are recently published and might extent the therapeutical spectrum [49, 50]. Moreover, few pharmaceutical treatments are thought to have an impact on plaque characteristics. The differences in medication in our study population therefore might also have an influence on plaque structure. The lipid lowering potential of statins is well known. Apparently these drugs are also capable of changes in the surface characteristics of plaque resident macrophages.

Effects on lowering CD36 and MRS1 expression, both macrophage scavenger receptors, were shown for atorvastatin by switching macrophages to a more antiatherogenic phenotype. Whereas increased expression of these receptors could be found in acute coronary syndrome and all in all seems to be associated with disease progression [51].

In our study, we did not find any effects on the number of osteogenic cells or plaque characteristics for aspirin as well as for a lipid lowering treatment. Additionally, we tested the correlation between plaque characteristics and cholesterol or LDL, respectively, without any statistical significance. However, we found a slight trend to a negative association between LDL and plaque size and calcification, respectively, in the target lesion ($r=-0,36$, $p=0,09$ and $r=-0,37$, $p=0,08$, respectively; data not shown). Since these subgroups include just small numbers of study subjects the statistical analyses remains limited.

One can argue about the use of mean area or volumetric data. Since we had quite a high spread of the analyzed segment lengths, we wanted to account for the different length of the analyzed segments by using the average instead of the total volume. For this reason there is only the correlation shown for the average area. Indeed, the correlation with volumetric data instead of the average area showed a similar tendency.

Regarding the way for detection of plaque components there is still a debate, since several studies showed contradicting results in term of correlation to the pathology [52]. Multimodality imaging including OCT might add to the detection of the plaque components. At the beginning of the study there was no OCT available in our lab. Therefore, we don't have this additionally modality. Keeping these limitations in mind, we found in our study at least the association with a plaque structure typical for vulnerable lesions

5. Conclusion

In this study, we show for the first time that retention of circulating osteogenic monocytes is associated with a greater extent of necrotic core and calcification in the early stage of coronary plaque development. Further studies are required to prove that the association between systemic levels and retention of osteogenic monocytes and markers of plaque destabilization implicate these cells in pathological remodeling of the coronary circulation in patients with early coronary atherosclerosis. Therefore these finding would make a substantial contribution in understanding the role of osteogenic monocytes in early plaque development.

Acknowledgments

This study was supported by National Institutes of Health grants HL92954, AG31750, HL77131.

The authors would like to thank Rebecca E. Nelson for helping with patient recruitment and Jonella Tilford with IVUS.

Abbreviations

APV Average peak velocity

BAP	Bone alkaline phosphatase
ED	Endothelial dysfunction
LAD	Left anterior descending artery
MACE	Major adverse cardiac event
NC	Necrotic core
OCN	Osteocalcin
TL	Target lesion
TS	Total segment
VH-IVUS	Virtual histology-intravascular ultrasound

References

1. Ling MY, et al. Up-regulated ATP-sensitive potassium channels play a role in increased inflammation and plaque vulnerability in macrophages. *Atherosclerosis*. 2013; 226(2):348–55. [PubMed: 23218803]
2. Beekhuizen H, et al. CD14 contributes to the adherence of human monocytes to cytokine-stimulated endothelial cells. *J Immunol*. 1991; 147(11):3761–7. [PubMed: 1719086]
3. Fadini GP, et al. Widespread increase in myeloid calcifying cells contributes to ectopic vascular calcification in type 2 diabetes. *Circ Res*. 2011; 108(9):1112–21. [PubMed: 21393578]
4. Nair A, et al. Coronary plaque classification with intravascular ultrasound radiofrequency data analysis. *Circulation*. 2002; 106(17):2200–6. [PubMed: 12390948]
5. Zamani P, et al. Relationship of antihypertensive treatment to plasma markers of vascular inflammation and remodeling in the Comparison of Amlodipine versus Enalapril to Limit Occurrences of Thrombosis study. *Am Heart J*. 2012; 163(4):735–40. [PubMed: 22520542]
6. Diethrich EB, et al. Virtual histology intravascular ultrasound assessment of carotid artery disease: the Carotid Artery Plaque Virtual Histology Evaluation (CAPITAL) study. *J Endovasc Ther*. 2007; 14(5):676–86. [PubMed: 17924734]
7. Lavi S, et al. Segmental coronary endothelial dysfunction in patients with minimal atherosclerosis is associated with necrotic core plaques. *Heart*. 2009; 95(18):1525–30. [PubMed: 19497916]
8. Yoon MH, et al. Long-term endothelin receptor antagonism attenuates coronary plaque progression in patients with early atherosclerosis. *Int J Cardiol*. 2013
9. Reriani M, et al. Long-term administration of endothelin receptor antagonist improves coronary endothelial function in patients with early atherosclerosis. *Circulation*. 2010; 122(10):958–66. [PubMed: 20733096]
10. Gossel M, et al. Coronary endothelial dysfunction in humans is associated with coronary retention of osteogenic endothelial progenitor cells. *Eur Heart J*. 2010; 31(23):2909–14. [PubMed: 20935001]
11. Lavi S, et al. The interaction between coronary endothelial dysfunction, local oxidative stress, and endogenous nitric oxide in humans. *Hypertension*. 2008; 51(1):127–33. [PubMed: 18086957]
12. Lavi S, et al. Local production of lipoprotein-associated phospholipase A2 and lysophosphatidylcholine in the coronary circulation: association with early coronary atherosclerosis and endothelial dysfunction in humans. *Circulation*. 2007; 115(21):2715–21. [PubMed: 17502572]
13. Gossel M, et al. Coronary endothelial dysfunction in humans is associated with coronary retention of osteogenic endothelial progenitor cells. *European heart journal*. 2010; 31(23):2909–14. [PubMed: 20935001]

14. Eghbali-Fatourehchi GZ, et al. Characterization of circulating osteoblast lineage cells in humans. *Bone*. 2007; 40(5):1370–7. [PubMed: 17320497]
15. Flammer AJ, et al. Patients with an HbA1c in the prediabetic and diabetic range have higher numbers of circulating cells with osteogenic and endothelial progenitor cell markers. *The Journal of clinical endocrinology and metabolism*. 2012; 97(12):4761–8. [PubMed: 23015657]
16. Eirin A, et al. Inflammatory and injury signals released from the post-stenotic human kidney. *European heart journal*. 2013; 34(7):540–548a. [PubMed: 22771675]
17. Flammer AJ, et al. Patients with an HbA1c in the prediabetic and diabetic range have higher numbers of circulating cells with osteogenic and endothelial progenitor cell markers. *J Clin Endocrinol Metab*. 2012; 97(12):4761–8. [PubMed: 23015657]
18. Eirin A, et al. Inflammatory and injury signals released from the post-stenotic human kidney. *Eur Heart J*. 2013; 34(7):540–548a. [PubMed: 22771675]
19. Matsuo Y, et al. Plaque characteristics and arterial remodeling in coronary and peripheral arterial systems. *Atherosclerosis*. 2012; 223(2):365–71. [PubMed: 22721702]
20. Lansky AJ, et al. Gender and the extent of coronary atherosclerosis, plaque composition, and clinical outcomes in acute coronary syndromes. *JACC Cardiovasc Imaging*. 2012; 5(3 Suppl):S62–72. [PubMed: 22421232]
21. Bae JH, et al. Association of angiotensin-converting enzyme inhibitors and serum lipids with plaque regression in cardiac allograft vasculopathy. *Transplantation*. 2006; 82(8):1108–11. [PubMed: 17060862]
22. Nair A, et al. Automated coronary plaque characterisation with intravascular ultrasound backscatter: ex vivo validation. *EuroIntervention*. 2007; 3(1):113–20. [PubMed: 19737694]
23. Han SH, et al. Rosuvastatin combined with ramipril significantly reduced atheroma volume by anti-inflammatory mechanism: comparative analysis with rosuvastatin alone by intravascular ultrasound. *Int J Cardiol*. 2012; 158(2):217–24. [PubMed: 21295363]
24. Ley K, Miller YI, Hedrick CC. Monocyte and macrophage dynamics during atherogenesis. *Arterioscler Thromb Vasc Biol*. 2011; 31(7):1506–16. [PubMed: 21677293]
25. Foresta C, et al. Platelets express and release osteocalcin and co-localize in human calcified atherosclerotic plaques. *J Thromb Haemost*. 2013; 11(2):357–65. [PubMed: 23206207]
26. Llodra J, et al. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci U S A*. 2004; 101(32):11779–84. [PubMed: 15280540]
27. Psaltis PJ, et al. Identification of a monocyte-predisposed hierarchy of hematopoietic progenitor cells in the adventitia of postnatal murine aorta. *Circulation*. 2012; 125(4):592–603. [PubMed: 22203692]
28. Tang Z, et al. Differentiation of multipotent vascular stem cells contributes to vascular diseases. *Nat Commun*. 2012; 3:875. [PubMed: 22673902]
29. Hu Y, et al. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *J Clin Invest*. 2004; 113(9):1258–65. [PubMed: 15124016]
30. Hu Y, Xu Q. Adventitial biology: differentiation and function. *Arterioscler Thromb Vasc Biol*. 2011; 31(7):1523–9. [PubMed: 21677295]
31. Massberg S, et al. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell*. 2007; 131(5):994–1008. [PubMed: 18045540]
32. Tavian M, et al. The vascular wall as a source of stem cells. *Ann N Y Acad Sci*. 2005; 1044:41–50. [PubMed: 15958696]
33. Heidt T, et al. Differential contribution of monocytes to heart macrophages in steady-state and after myocardial infarction. *Circulation research*. 2014; 115(2):284–95. [PubMed: 24786973]
34. Hashimoto D, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*. 2013; 38(4):792–804. [PubMed: 23601688]
35. Teraa M, et al. Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. *PloS one*. 2013; 8(1):e55592. [PubMed: 23383236]

36. Teraa M, et al. Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. *PLoS One*. 2013; 8(1):e55592. [PubMed: 23383236]
37. Tilki D, et al. Emerging biology of vascular wall progenitor cells in health and disease. *Trends Mol Med*. 2009; 15(11):501–9. [PubMed: 19828379]
38. Mestas J, Ley K. Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc Med*. 2008; 18(6):228–32. [PubMed: 19185814]
39. Gautier EL, Jakubzick C, Randolph GJ. Regulation of the migration and survival of monocyte subsets by chemokine receptors and its relevance to atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2009; 29(10):1412–8. [PubMed: 19759373]
40. Seimon T, Tabas I. Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J Lipid Res*. 2009; 50(Suppl):S382–7. [PubMed: 18953058]
41. Schrijvers DM, et al. Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability. *Cardiovasc Res*. 2007; 73(3):470–80. [PubMed: 17084825]
42. Vittone F, et al. Sitagliptin reduces plaque macrophage content and stabilises arteriosclerotic lesions in Apoe (–/–) mice. *Diabetologia*. 2012; 55(8):2267–75. [PubMed: 22648661]
43. Guihard P, et al. Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling. *Stem cells*. 2012; 30(4):762–72. [PubMed: 22267310]
44. Aikawa E, et al. Arterial and aortic valve calcification abolished by elastolytic cathepsin S deficiency in chronic renal disease. *Circulation*. 2009; 119(13):1785–94. [PubMed: 19307473]
45. Sakakura K, et al. Pathophysiology of atherosclerosis plaque progression. *Heart Lung Circ*. 2013; 22(6):399–411. [PubMed: 23541627]
46. Schlett CL, et al. Prognostic value of CT angiography for major adverse cardiac events in patients with acute chest pain from the emergency department: 2-year outcomes of the ROMICAT trial. *JACC. Cardiovascular imaging*. 2011; 4(5):481–91. [PubMed: 21565735]
47. D'Ascenzo F, et al. Atherosclerotic coronary plaque regression and the risk of adverse cardiovascular events: a meta-regression of randomized clinical trials. *Atherosclerosis*. 2013; 226(1):178–85. [PubMed: 23206978]
48. Maldonado N, et al. A mechanistic analysis of the role of microcalcifications in atherosclerotic plaque stability: potential implications for plaque rupture. *Am J Physiol Heart Circ Physiol*. 2012; 303(5):H619–28. [PubMed: 22777419]
49. Zhi K, et al. alpha4beta7 Integrin (LPAM-1) is Upregulated at Atherosclerotic Lesions and is Involved in Atherosclerosis Progression. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. 2014; 33(6):1876–87.
50. Petersen LK, et al. Amphiphilic nanoparticles repress macrophage atherogenesis: novel core/shell designs for scavenger receptor targeting and down-regulation. *Molecular pharmaceutics*. 2014; 11(8):2815–24. [PubMed: 24972372]
51. Piechota M, et al. Highly upregulated expression of CD36 and MSR1 in circulating monocytes of patients with acute coronary syndromes. *The protein journal*. 2012; 31(6):511–8. [PubMed: 22763563]
52. Falk E, et al. Update on acute coronary syndromes: the pathologists' view. *Eur Heart J*. 2013; 34(10):719–28. [PubMed: 23242196]

Highlights

- CD14+/BAP+/OCN+ monocytes associate with remodeling of coronary circulation.
- CD14+/BAP+/OCN+ monocytes may be associated with plaque destabilization.
- Circulating CD14+/BAP+/OCN+ monocytes correlate with necrotic core and calcification.
- Coronary retention of CD14+/OCN+ cells correlate with necrotic core and calcification.

Figure 1.

Example for flow analysis. **A** Shows the forward/side scatter and the gate used in the analysis. Panel **B** and **C** show the staining with isotype controls for the CD14- and Osteocalcin (OCN)-specific antibodies and bone alkaline phosphatase (BAP), respectively, and their appropriate antibodies (**D**, **E**). All are referred to R1. Panel **E** shows the CD14+/OCN+ cells stained with the BAP-antibody and refers to R2. Threshold for isotype controls was required < 0.3% (<1.0% for OCN). APC, Allophycocyanin; FITC, Fluorescein isothiocyanate.

Fig. 2a

Fig. 2b

Fig. 2c

Figure 2.

Figure 2a,b,c. Diagram for assessment of the plaque target lesion. Target lesions set to 10mm were defined as distal 5mm and proximal 5mm from the slice of the lesion with the greatest plaque burden.

Fig. 3a

Fig. 3b

Figure 3. Distribution of plaque burden in the total segment and the target lesion of the 23 analyzed segments.

Figure 4.

Correlation of net gradient of CD14+/OCN+ monocytes with average area of necrotic core and calcification in the target lesion. For the statistical comparison Spearman's coefficient was used with continuous variables. The group-based presentation was chosen for illustrating the distinct skewed data.

Figure 5.

Correlation of circulating CD14+/BAP+/OCN+-monocytes with average necrotic core area and calcification in the target lesion. For the statistical comparison Spearman's coefficient was used with continuous variables. The group-based presentation was chosen for illustrating the distinct skewed data.

Table 1

Patient demographics.

	Study patients (n=23)
Age	51.5 ± 9.0
Sex (male, %)	62
BMI (kg/m ²)	28.9 ± 5.5
sBP (mmHg)	119.6 ± 14.8
dBP (mmHg)	73 ± 10.0
Heart rate (per min)	68.2 ± 11.4
Hypertension [n (%)]	12 (52)
CV family history [n (%)]	12 (52)
Diabetes mellitus [n (%)]	2 (9)
Obesity [BMI > 30; n (%)]	8 (35)
Smoking history [n (%)]	9 (39)
<u>CV drug use:</u>	
Aspirin [n (%)]	15 (65)
ACE inhibitor [n (%)]	2 (9)
Beta blocker [n (%)]	6 (26)
CCB [n (%)]	11 (48)
Lipid lowering drug [n (%)]	5 (22)

Values are mean ± standard deviation. P-values <0.05 were considered significant and marked bold. BMI, body mass index; sBP, systolic blood pressure; dBP diastolic blood pressure; RF, risk factor; CV, cardiovascular; ACE, angiotensin-converting enzyme; CCB, calcium channel blocker

Table 2

IVUS characteristics and VH-IVUS data in the study group (n=23).

	Total segment		Target lesion
Segment length (mm)	59.4 ± 24.9		10
Lumen area (mm ²) *	9.1 ± 2.4		9.7 ± 3.6
Minimal diameter lumen (mm)	2.4 ± 0.4		2.9 ± 0.6
Maximal diameter lumen (mm)	4.8 ± 0.7		4.1 ± 0.7
Vessel area (mm ²) *	12.1 ± 3.0		13.7 ± 4.3
Minimal diameter vessel (mm)	2.8 ± 0.5		3.6 ± 0.7
Maximal diameter vessel (mm)	5.3 ± 0.8		4.8 ± 0.7

	Volume (mm ³)	Average area (mm ²) *	Volume (mm ³)	Average area (mm ²) *
Plaque burden *	190.92 ± 130.56	3.0 ± 1.2	40.01 ± 17.50	4.0 ± 1.8
<u>VH-IVUS</u>				
Dense calcium	4.67 ± 8.80	0.06 ± 0.11	2.13 ± 3.84	0.21 ± 0.39
Necrotic core	7.96 ± 12.69	0.11 ± 0.15	2.66 ± 3.99	0.26 ± 0.40
Fibrous	27.88 ± 37.46	0.40 ± 0.50	5.55 ± 5.42	0.56 ± 0.55
Fibro-fatty	27.89 ± 37.46	0.06 ± 0.08	0.67 ± 0.73	0.07 ± 0.07

Values are mean ± standard deviation. VH-IVUS, virtual histology-intravascular ultrasound;

* Values are all normalized for length

Table 3

Correlation between monocyte subsets and coronary imaging (after normalization to white blood cell count).

	Circulating CD14+/BAP+/OCN+ cells		Net gradient CD14+/OCN+ cells	
<i>Calcification</i>	r=0.55,	p=0.006	r=0.61,	p=0.003
<i>Necrotic core</i>	r=0.53,	p=0.010	r=0.61,	p=0.003

Shown are Spearman coefficients (r) with associated p-values. Row p-values indicate an overall test for association between the imaging measure and the monocyte measures. Row p-values were significant < 0.005 (to account for multiple tests) before individually tested correlations. Within significant rows, individual p-values were significant < 0.01.

OCN, osteocalcin; BAP, bone alkaline phosphatase; TS, total segment; TL, target lesion