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Monocyte subsets in coronary artery disease and their associations with markers of inflammation and fibrinolysis

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ABSTRACT

Aims: The multiple roles of monocytes in atherogenesis, including inflammation, angiogenesis and repair are attributed to the existence of different monocyte sub-populations. Scarce data are available on changes in phenotype and functional status of human monocyte subsets in patients with coronary artery disease (CAD), especially when monocytes are evaluated as three distinct subsets.

Methods and results: Surface expression of receptors implicated in inflammation, repair and activation status (intracellular IKKβ) of monocyte subsets was assessed by flow cytometry in 53 patients with CAD and compared to 50 age- and sex-matched healthy controls. Monocyte subsets were defined as CD14++CD16−CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2), and CD14+CD16++CCR2− (Mon3). Plasma levels of inflammatory cytokines (FACSArray) and fibrinolytic factors (ELISA) were measured in CAD. CAD was associated with reduced expression of CD14 on Mon1 ($p = 0.02$) and Mon3 ($p = 0.036$), higher expression of IL6 receptor on Mon1 ($p = 0.025$) and Mon2 ($p = 0.015$), CXCR4 on Mon1 ($p = 0.035$) and Mon3 ($p = 0.003$), and CD34 on all subsets (all $p < 0.007$). Monocyte CD163 expression correlated negatively with interleukin (IL)-6 levels ($p < 0.01$ for all subsets). Expression of vascular endothelial growth factor receptor-1 correlated positively with plasminogen activator inhibitor (PAI)-1 antigen levels ($r = 0.47$, $p = 0.006$). In vitro, monocyte subsets derived from CAD patients showed significantly altered responses to endotoxin stimulation compared to monocytes from healthy controls.

Conclusions: There is a complex interplay between phenotype and activity of monocytes and plasma cytokines and fibrinolytic factors. These findings support the presence of unique roles for the three human monocyte subsets in atherogenesis and CAD pathogenesis.

1. Introduction

Monocyte-derived 'foam' cells have been established as a hallmark and key pathogenic feature of atherosclerosis, a disease of inflammatory aetiology [1–3]. High total monocyte count is a strong predictor of high risk of coronary artery disease (CAD) or myocardial infarction (MI) [4]. However the roles of monocytes in atherosclerosis are diverse, including involvement in inflammatory responses and the regulation of thrombogenic status (e.g., via tissue factor expression and modulation of fibrinolysis). Indeed, monocytes have also been implicated in physiologically beneficial processes related to scavenging of redundant/pathological material, angiogenesis and repair [5–7].

This diversity of monocyte functions can be partly attributed to the existence of different monocyte subsets distinguished by specific phenotypic and functional properties [8–13]. Specific monocyte subsets are differentially involved in the pathogenesis and outcome of acute coronary syndromes, heart failure, and stroke, amongst other conditions [14,15]. However, most previous studies in both rodents and humans are limited by the analysis of monocytes as only two subsets, where CD16+ cells are considered as a single population, in contract to the current monocyte nomenclature.

The aim of this study was to provide a detailed phenotypic comparison of the three human monocyte subsets defined according to contemporary nomenclature between patients with CAD and healthy subjects, with a focus on investigating differences in expression of receptors involved in inflammatory and reparative responses between subsets. Additionally, we provide an insight into monocyte functional status, by measuring intracellular IKKβ

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(IκB kinase β, a marker of activation of nuclear factor κB [NFκB] pathway), quantify monocyte interactions with platelets, and evaluate association of monocyte characteristics with plasma markers of inflammation and fibrinolysis.

2. Methods

We recruited 53 patients with stable CAD confirmed during elective coronary angiography on contemporary medical therapy with no hospital admissions for ≥3 months and free from angina at the time of study (referred to Sandwell and West Birmingham Hospitals NHS Trust). Exclusion criteria comprised factors known to affect monocyte count (infectious disease, inflammatory disorders and their treatment, cancer, haemodynamically significant valvular heart disease, atrial fibrillation, renal failure and hormone replacement therapy). Patients with CAD were compared to 50 'healthy controls' with no history of cardiovascular or other chronic morbidity, based on history and clinical examination.

Peripheral venous blood samples were collected from all participants and processed by flow cytometry within 60 min for assessment of monocyte characteristics. Plasma was stored at −70 °C for batched analysis. The study was performed in accordance with the Helsinki declaration and was approved by the Coventry Research Ethics Committee. All participants provided written informed consent.

2.1. Flow cytometry

Flow cytometric analysis was performed using the BD FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK [BD]) as previously described [16]. The technique is robust and highly reproducible. The laboratory coefficient of variation for absolute monocyte count is 1.9%, and for surface and intracellular markers is <5%.

(i) Absolute count of monocyte subsets and monocyte platelet aggregates

Mouse anti-human monoclonal fluorochrome-conjugated antibodies anti-CD16-Alexa Fluor 488 (AbD Serotec, Oxford, UK), anti-CD14-PE (BD), anti-CD42a-PerCP (BD) and anti-CCR2-APC (R&D Systems, Abingdon, UK [RD]) were mixed with 50 μl of fresh EDTA anticoagulated whole blood in TruCount tubes (BD) containing a strictly defined number of fluorescent count beads. After incubation for 15 min, red blood cells were lysed by 450 μl of lysing solution® (BD) for 15 min, followed by dilution in 1.5 ml of phosphate buffered saline and immediate flow cytometric analysis. Monocyte subsets were defined as CD14++CD16−CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2− (Mon3) in accordance with contemporary nomenclature [17]. Absolute count of monocyte subsets was obtained using the count beads according to the manufacturer's recommendations. Monocyte-platelet aggregates (MPAs) were defined as events positive to both monocyte markers and the platelet marker CD42a (glycoprotein IX).

(ii) Expression of surface antigens on monocyte subsets

For analysis of surface antigens, 100 μl of whole blood was incubated with mouse anti-human monoclonal flouorochrome-conjugated antibodies for 15 min in the dark. Red blood cells were lysed with 2 ml of BD lysing solution® for 10 min, washed in PBS followed by immediate flow cytometric analysis. Anti-CD16-Alexa Fluor 488 (AbD Serotec, Oxford, UK) and anti-CD14-PerCP-Cy5.5 (BD) were used for definition of monocyte subsets into CD14++CD16− monocytes (Mon1), CD14++CD16+ monocytes (Mon2) and CD14+CD16++ monocytes (Mon3). PE-conjugated antibodies were used against Toll-like receptor-4 (TLR4, clone 285219, R&D), integrin β2/CD18 (intracellular adhesion molecule (ICAM) receptor, R&D Systems Europe Ltd., Abingdon, UK [R&D]), CXCR4 (stromal cell-derived factor-1 [SDF1] receptor, R&D), CD34 (BD), vascular endothelium growth factor (VEGF) receptor 1 (R&D). APC-conjugated antibodies were used against interleukin (IL)6 receptor (IL6R, R&D), integrin α4/CD49d (vascular cells adhesion molecule-1 [VCAM] receptor, R&D), CD163 (R&D), KDR (VEGF receptor 2, R&D), CD204 (R&D). Monoclonal antibodies for CD204 were conjugated to APC using LL-APC-XL conjugation kit [Innova Biosciences, UK]. Expression of the surface markers was quantified as median fluorescence intensity (MFI).

(iii) Assessment of intracellular activation of nuclear factor κB pathway

Intracellular level of IKKβ, a cytoplasmic marker of activation of the NFκB pathway, was measured from fresh whole blood [16]. 100 μl of blood was incubated with monoclonal mouse anti-human antibodies against CD16-Alexa Fluor 488 (AbD Serotec, Oxford, UK) and CD14-PerCP-Cy5.5 (BD) for 15 min followed by lysing of red blood cells with 2 ml of BD PharmLyse™ for 10 min and washing in staining buffer. The resulting pellet was resuspended in fixation/permeabilization solution (BD) for 20 min and, following centrifugation, in 2 ml of BD Perm/Wash™ buffer for 10 min. Following further centrifugation, the pellet was incubated for 30 min with monoclonal mouse anti-human APC-conjugated antibodies (LL-APC-XL conjugation kit [Innova Biosciences, UK]) against IKKβ (clone 10A9B6, Abcam, Cambridge, UK), washed and resuspended in 200 μl of 2% PBS/2% paraformaldehyde solution (PharmFix, BD) for immediate flow cytometric analysis. IKKβ values were quantified by their MFI.

(iv) Plasma markers

Plasma levels of IL-1β, IL-6, IL-10 and monocyte chemoattractant protein (MCP-1) were measured by cytometric bead array technology. The BD FACSCalibur flow cytometer was used for data acquisition, with FCAP Array v2.0.2 software (Burnsville, Minnesota, USA) for data analysis. Commercially available reagent sets, Human IL-1β Flex Set, Human IL-6 Flex Set, Human IL-10 Flex Set and Human MCP-1 Flex Set (all from BD) were used according to the manufacturer's recommendations. The inter- and intra-assay coefficients of variation for all assays were <5%.

Enzyme-linked immunosorbent assay (ELISA) of citrated plasma was used to quantify levels and activity of fibrinolytic parameters. All measurements were performed using high quality ZYMUTEST kits (Hyphen Bio-Med, Neuville-sur-Oise, France): tissue type plasminogen activator (tPA) antigen, plasminogen activator inhibitor type 1 (PAI-1) antigen, PAI-1 activity and urikinase type plasminogen activator (uPA) antigen. Measurements were performed according to the manufacturer's recommendations [7].

2.2. Isolation and functional assessment of monocyte subsets

MFI of six intracellular inflammatory markers, including IKKβ, was assessed in each monocyte subpopulation pre- and post-endotoxin, as indexes of monocyte function. Thirty ml of anticoagulated blood was collected from 5 patients with stable CAD and 3 healthy volunteers. Monocyte subpopulations were isolated from whole blood as previously described [16]. In brief, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll−Plaque density gradient centrifugation. Monocyte subsets were further isolated by