Co-culture of platelets with monocytes induced M2 macrophage polarization and formation of foam cells: shedding light on the crucial role of platelets in monocyte differentiation.

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Abstract

Objectives: Far beyond hemostasis and thrombosis, a growing body of evidence indicated the critical role of platelets in atherosclerosis. SDF-1 is among pro-inflammatory chemokines which is increased on platelets of patients with coronary artery disease (CAD). In the current study, we aimed to investigate the in vitro effect of platelets, either from CAD patients or healthy volunteers, on the induction of macrophages and foam cells.

Materials and Methods: The surface expression of SDF-1 on platelets of CAD patients and healthy volunteers was investigated using flow cytometry. We also evaluated the CXCR4/CXCR7 expression on monocytes from buffy coats of healthy volunteers. The effect of platelets from CAD patients and healthy volunteers on differentiation of monocytes and foam cells formation was evaluated using oil red O (ORO) staining. Flow cytometry and real time PCR were also employed to evaluate surface markers and mRNA expression of genes involved in this process after co-culture of platelets with monocytes.

Results: Monocytes in co-culture with platelets acquired a spindled shape appearance and ORO-positive lipid droplets. In addition, platelets could induce CD163 expression, as an important marker of M2 macrophage, and upregulate the mRNA expression of SRB, CD36, ACAT, LXR-α, and ABCA1 genes in monocytes. Notably, platelets of CAD patients with higher surface expression of SDF-1, increased the expression of genes encoding SRB and CD36 as compared to platelets of healthy volunteers.

Conclusion: Our results indicate that platelets from CAD patients could provoke monocyte differentiation into macrophages with a M2 phenotype, which in turn may participate in an atheroprotective process.

Key words: SDF-1, monocytes, platelets, co-culture, foam cells.
1. Introduction

Atherosclerosis is the most common pathological process leading to cardiovascular diseases and involves a complex interaction between cellular and non-cellular constituents which include platelets, cytokines and leukocytes [1]. Far beyond hemostasis and thrombosis, the critical role of platelets in wound healing, inflammation, atherosclerosis and immunity system has been recognized [2, 3]. Platelets store a wide variety of cytokines, chemokines, mitogens, angiogenic factors and other bioactive molecules in their granules [4, 5] which can potentially induce pro- and anti-inflammatory responses [6]. Several investigations have reported the crucial role of chemokines in the pathogenesis of cardiovascular diseases [7]. SDF-1 (CXCL12), is among pro-inflammatory chemokine which is released following platelet activation [8, 9]. Expression of SDF-1 on circulating platelets is increased in patients with coronary artery disease (CAD) [10, 11] which is associated with cardio-protection and improved left ventricular function after myocardial infarction [12, 13]. At injury sites, platelets recruit and activate leukocytes through cell-cell interactions or indirectly by release of their mediators [14]. Remarkably, platelet-derived SDF-1 can bind to its chemokine receptors, CXCR4 and CXCR7 on monocytes and facilitate phagocytosis of apoptotic platelets by these cells and promote their differentiation into foam cells [15].

Classically, activated macrophages are categorized into pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages [16]. Briefly, M1 macrophages sustain the inflammatory response through production of inflammatory cytokines. M2 macrophages, on the other hand, are involved in tissue repair and wound healing [17]. According to the expression of surface markers, CD86 is used as M1 macrophage marker [18], while, CD163, is commonly used to detect M2 macrophages [19].

We hypothesized that platelets of patients with CAD, which have increased expression of SDF-1, will promote monocyte differentiation into macrophages and foam cell. Therefore, the aims of this study were to investigate the in vitro effect of CAD patients platelets in induction of macrophage and foam cells in comparison with normal platelets.

2. Materials and Methods

2.1. Materials

Mouse monoclonal anti-human CD61-FITC (Dako, Denmark), mouse monoclonal anti-human CXCR4-PE (Biolegend, USA), mouse monoclonal anti human/mouse CXCR7-PE (Biolegend, USA), mouse monoclonal anti-human SDF-1- PerCP (Novus Biologicals, USA), mouse monoclonal anti-human CD14-PE (Beckman Coulter, USA), mouse monoclonal anti-human CD11b-PE (Dako, Denmark), mouse monoclonal anti-human CD11c-FITC (Dako, Denmark), mouse monoclonal anti human CD86-PerCP (Abcam, USA) and mouse monoclonal anti-human CD163-FITC (R&D Systems, UK) were used in our study. Ficoll-Hypaque was from Lymphodex (inno TRA i n, Germany), Taq DNA Polymerase 2x Master Mix RED and Real Q Plus Master mix Green Low ROX™ were procured from Ampliqon (Copenhagen, Denmark), RevertAid First Strand eDNA Synthesis Kit from Thermo Fisher Scientific (USA) and Trypan blue, Oil red O (ORO) stain and TRIzol were purchased from Sigma-Aldrich (USA).

2.2. Expression of SDF-1 on platelets of CAD patients and healthy volunteers

Ten patients with symptomatic CAD who were visited at the cardiovascular section of Taleghani hospital (Tehran) were investigated for platelet surface expression of SDF-1. CAD patients were selected with respect to clinical symptoms, myocardial ischemia markers and ECG. The
increased level of SDF-1 was as inclusion criteria for selection of CAD patients for evaluation in co-culture, while exclusion criteria were diabetes, history of CAD in family, hypertension, hyperlipidemia, smoking and medication on admission. Ten healthy volunteers with no history of cardiovascular diseases which were matched with CAD patients regarding age and sex (P>0.05) were also evaluated as normal controls. At last, three patients with high expression of this marker were selected for platelets preparation in comparison with three healthy volunteers with low expression of SDF-1. This study was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR. SBMU. RETECH.REC. 1396.717) and all the participants gave informed consent in accordance with the deceleration of Helsinki. To detect the surface expression of SDF-1, diluted PRP was incubated with mouse monoclonal anti-human SDF-1-PerCP and mouse monoclonal anti-human CD61-FITC or their respective isotype controls and analyzed by flow cytometer (Attune NxT; Life Technologies, USA). CD61-FITC was used to identify the platelet population and mean fluorescence intensity (MFI) was used as a quantitative indicator for the surface expression of the indicated protein.

2.3. Isolation of platelets

Blood was collected in ACD-A (Acid Citrate Dextrose solution-A) anticoagulant (1:4) from coronary artery disease (CAD) patients and healthy volunteers and centrifuged at 200 × g for 15 min. The platelet-rich plasma (PRP) thus obtained was added to Tyrodes-HEPES buffer (HEPES-2.5 mM; NaCl-150 mM; KCl-1 mM; NaHCO3-2.5 mM; NaH2PO4-0.36 mM; glucose-5.5 mM; BSA-1 mg/ml; pH 6.5) and centrifuged at 800 × g for 10 min. The platelet pellet was suspended in Tyrodes-HEPES buffer (pH 7.4; supplemented with CaCl2-1 mM; MgCl2-1 mM).

2.4. Isolation of peripheral blood monocytes

Leukocyte buffy coat preparations from healthy volunteers were used for isolation of peripheral blood monocytes through differential gradient centrifugation in Ficoll-Hypaque gradient (20 min, 760 × g), followed by adhesion depletion on plastic surface. Non-adherent cells were removed by gentle washing after 4 h and the remaining adherent cells (monocytes) were harvested. Viability of monocytes, as assessed by Trypan blue dye exclusion, was >98% immediately after their isolation. Purity of isolated monocytes was assessed by FSC-SSC parameters and surface expression of CD14 by flow cytometry. The expression of CXCR4 and CXCR7 on monocytes were also evaluated by flow cytometry.

2.5. Monocyte and platelet co-culture

Monocytes were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37 °C and 5% CO2 humidified atmosphere. Isolated platelets were added to monocytes (monocyte to platelet 1:100), and half of the culture medium was exchanged with fresh complete medium every 2 days. During co-culture, monocytes were examined by microscopic analysis. After co-culture, platelets or floating cells were washed away and monocyte analyzed by ORO staining, flow cytometry and real time PCR.

2.6. ORO staining for foam cells evaluation

For ORO staining, the cells were stained after 7, 10 and 15 days of co-culture. After 2-3 times washing with PBS, cells were fixed with 10% formalin for 30 min, penetrated with 60% isopropanol for 5 min, and stained with ORO staining for 10 minutes at room temperature. To remove any residual ORO stain, stained wells were destained with 60% isopropanol for 15 s and then washed with PBS for 3 times. Images of foam cells were taken under 10× and 40×
objectives in the light microscope (Olympus) with the Optika camera (Italy). Image analysis was performed using ImageJ software.

2.7. Flow cytometry assessment of monocytes
Surface expression of markers on differentiated macrophages were analyzed using flow cytometry following 7 days co-culture. Cells were harvested, washed in PBS and labeled with fluorescent-conjugated antibodies against CD14, CD11b, CD11c, CD86, CD163 or their respective isotype controls, then incubated for 30 min at 4 °C in the dark. Thereafter, the cells were fixed with 0.5% paraformaldehyde and were measured using flow cytometry. Analysis of data was performed by gating on viable cells using FSC-SSC characteristics in the monocyte/macrophage population. The percentage of each marker is determined against its respective isotype control.

2.8. Quantitative Real-Time PCR (qRT-PCR)
Total RNAs were isolated from harvested monocytes following 7 days co-culture with TRIzol according to the manufacturer’s recommendation and converted to complementary DNA (cDNA) using the cDNA synthesis kit. Quantitative Real-time PCR for ABCA1, CD36, SRB, PPARγ, SRA, ACAT1, ABCG1 and LXR-α (NR1H3) was carried out on Rotor Gene Q (QIAGEN, Germany) using Real Time PCR Master Mix. Housekeeping gene ABL was also used as a control for differences in RNA concentrations. The primers used in this research are listed in Table1.

<table>
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<th>GENE</th>
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<th>Reverse Primer (5′-3′)</th>
<th>Product size (bp)</th>
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<td>CCGCAGAAAGATGTCACTCAACG</td>
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<tr>
<td>CD36</td>
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<tr>
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<tr>
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<td>GACGTAAGCTTGCCATCGAGAG</td>
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2.9. Statistical analysis
Each experimental condition was performed in triplicate and all data are presented as mean ± SD. Statistical analysis was performed using Prism software (GraphPad Software, San Diego, California). To determine the difference between two groups, unpaired two-tailed Student t-test was applied. Analysis of variance (ANOVA) was applied for multiple comparisons. In all cases, the minimum statistical significance was P< 0.05.
3. Results

3.1. Platelets of patients with coronary artery disease had increased expression of SDF-1

As shown in Fig. 1, analysis of flow cytometry data showed that the expression of SDF-1 on the platelets of CAD patients was significantly higher than healthy controls (MFI: 1112±304 vs. 943±131, P = 0.042).

![Fig. 1](image1)

**Fig. 1:** Assessment of the surface expression of SDF-1 on the platelets of ten healthy volunteers and ten patients with symptomatic CAD. (a) Surface expression of SDF-1 on the platelets of CAD patients was significantly higher than healthy controls. (b) The comparison of surface expression of SDF-1 on the platelets of controls and CAD patients. Student t-test was applied to determine the difference between two groups. Values are given as mean ± SD *: P-value< 0.05.

3.2. Surface expression of CXCR4 and CXCR7 on monocytes

We evaluated the surface expression of CXCR4 and CXCR7 on monocytes isolated from buffy coat on first day. Our results showed that the surface expression of CXCR4 and CXCR7 markers on monocytes was 51.7±3.2 and 28.5%±2.5, respectively (Fig. 2).

![Fig. 2](image2)

**Fig. 2:** Flow cytometry analysis of CXCR4 and CXCR7 on monocytes isolated from buffy coat. (a) Flow cytometric histograms showed the surface expression of CXCR4 and CXCR7 on monocytes isolated from buffy coats with respect to their isotype controls. (b) The comparison of surface expression of CXCR4 and CXCR7 on monocytes isolated from buffy coats. Experiments were conducted in triplicate.

3.3. Platelets significantly induced spindled shape appearance in macrophages
In vitro and in vivo maturation of blood monocytes into macrophages take place as a consequence of interactions with different cell types. We evaluated monocyte morphology during 20 days from the co-culture. In this study, we showed that co-culture of platelets with monocytes prompted platelet clearance and monocyte differentiation into macrophages with spindle-shaped appearance. After prolonged co-culture (15 days), substantial percentage of macrophages showed morphological features of foam cells. Moreover, our results indicated that platelets of CAD patients enhanced differentiation of monocytes to foam cells in comparison with platelets of healthy volunteers (Fig. 3).

Fig. 3: Changes assessment in monocyte morphology at different days of culture. (a) Platelets promoted differentiation of monocytes to macrophages and foam cells as compared to platelet free culture. Furthermore, platelets of CAD patients enhanced differentiation of monocytes to foam cells in comparison with platelets of healthy volunteers. Monocytes shape change following co-culture with platelets are shown with arrows in the picture. Magnification 400×. (b) Bar diagram representing a comparison of monocytes shape change during different days of cultures. Experiments were conducted in triplicate.

3.4. Oil Red O staining showed significant ORO-stained lipid droplets in monocytes
Formation of foam cells is time dependent, so we stained and analyzed ORO-stained lipid droplets on days 7, 10 and 15 following co-culture off monocytes and platelets. After 7 days, macrophages showed no significant ORO-stained lipid droplets. Although upon further prolonging the platelet-monocyte co-culture to 10 and 15 days, lipid droplets were seen more significantly in macrophages cultured with platelets. Furthermore, ORO staining indicated that platelets of CAD patients enhanced differentiation of monocytes into foam cells in comparison with platelets of healthy volunteers (Fig. 4).
Fig. 4: Evaluation of macrophage foam cells using Oil red O staining during different days of co-culture. (a) After 7 days, macrophages showed no significant ORO-stained lipid droplets, although after 10 and 15 days from co-culture, ORO-stained lipid droplets were seen more significantly in macrophage foam cells cultured with platelets. Furthermore, ORO staining indicated that platelets of CAD patients enhanced differentiation of monocytes to foam cells in comparison with platelets of healthy volunteers. Foam cells are shown with arrows in the picture. Magnification 400×. (b) Bar diagram representing a comparison of ORO positive cells between different samples on days 10 and 15. Each experiment was conducted in triplicate.

3.5. Platelets increased CD163 expression on monocytes after 7 days

CD163 has a low to moderate expression on monocytes [20], however, its high expression is seen on macrophages of the 'alternative activation' phenotype. These cells play an important role in inhibiting the inflammatory responses and scavenging components of damaged cells [21]. Flow cytometry conducted to detect the surface expression of monocytes markers following isolation. As is shown in Fig. 5-a, the purity of isolated monocytes was analyzed by FSC-SSC parameters and surface expression of CD14 was above 98%. Expression of other markers was analyzed on day 1 that high expression of CD11b and CD11c and low expression levels of CD163 and CD86 were identified. In monocytes on day 7, either were cultured in present of platelets or those cultured without platelets, high expression of CD14, CD11b and CD11c were detected (Fig. 5-b). CD86 expression on monocytes was associated with a decrease after 7 days from co-culture, independent of the presence or absence of platelets. A slight increased expression of CD163 was observed on platelet-free cultures on day 7 compared with day 1. However, a significant increase of this marker was detected on monocytes which co-cultured with platelets (Fig. 5-c).
Fig. 5: Platelets induced differentiation of monocytes into CD163+ macrophages. (a) Purity of isolated monocytes was ascertained via evaluation of CD14 by flow cytometry. (b) Bar diagram representing phenotypic characterization of monocyte in terms of CD14, CD11b and CD11c surface expression in co-culture with platelets compared with platelet-free cultures. (c) Data represent CD86 and CD163 expression of monocytes by flow cytometry. Flow cytometry histogram of the surface expression of CD86 and CD163 as compared to their respective isotype controls showing prominent surface expression of CD163 on monocyte in co-culture of platelets and monocytes. Bar diagram representing phenotypic characterization of monocyte in terms of CD86 and CD163 surface expression which shows a predominant CD163 expression on monocytes in co-culture with platelets compared with platelet-free cultures. CD86 expression on monocytes was associated with a decrease after 7 days from culture.

3.6. Platelets induced the mRNA expression of the genes involved in cholesterol absorption, cholesterol esterification and cholesterol efflux in monocytes

In this study, following co-culture of monocytes with platelets, we investigated the mRNA expression of CD36, scavenger receptor class A (SR-A) and acyl-coenzyme A cholesterol acyltransferase (ACAT) in monocytes, which are among key genes in lipid accumulation [22, 23]. On the other hand, we evaluated the gene expression changes which are expected to enhance the cholesterol efflux from foam cells including, ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1), peroxisome proliferator-activated receptor γ (PPARγ), Liver X receptor α (LXRα) and scavenger receptor class B (SRB) [24-26]. Our results showed that platelets significantly increased the mRNA expression of CD36, ACAT, ABCA1, SRB and LXR-α. Furthermore, platelets of CAD patients, increased the mRNA expression of CD36 and SRB more significantly than platelets of healthy volunteers which indicated that these platelets have a more profound role in formation of macrophage and foam cells (Fig. 6).
Fig. 6: Evaluation of gene expression changes of monocytes after 7 days. After co-culture of monocytes with platelets of healthy volunteers, analysis of qRT-PCR demonstrated the increased expression of following genes, SRB, CD36, ACAT, LXRα and ABCA1. Co-culture of monocytes with platelets of CAD patients had the same results, but as compared to platelets of healthy volunteers, increased expression of CD36 and SRB was observed. Values are given as mean ± SD of three independent experiments. *, P ≤ 0.05

4. Discussion

In inflammatory diseases such as atherosclerosis, platelets are important players and recruit the other cells towards the lesion sites [27]. Alpha-granules of platelets are major sources of SDF-1, a well-known chemokine which is over-expressed on the surface of platelets upon activation [28]. Notably, it has been reported that among leukocyte subtypes, monocytes preferentially interact with activated platelets through CXCR4 and CXCR7 [15]. According to the studies, surface expression of CD163, as an important marker of M2 macrophage, is elevated in macrophages of inflamed tissues such as atherosclerotic lesion [29]. Moreover, Buchacher et al. indicated that monocytes with the spindle-shape morphology are mainly representative of M2 macrophages [30]. In the present study, we investigated the in vitro interaction of monocytes and platelets during 20 days. We showed that during 7 days platelets not only drive differentiation of monocytes to the spindle-shape macrophages, but also up-regulate the expression of CD163, which is in agreement with a recent study conducted by Chatterjee et al [15]. Our results also revealed that the longer co-culture of the cells resulted in a considerable percentage of macrophages presenting morphological features of foam cells. It is worth mentioning that platelets of CAD patients which express a higher percentage of SDF-1 induced greater numbers of foam cells as compared to platelets of healthy volunteers; indicating that platelet-derived SDF-1 may play a probable role in differentiation of monocytes to foam cells.

Although previous studies discussed about the contributory role of platelet-derived SDF-1 in differentiation of monocytes into macrophage/foam cells [9], the precise molecular mechanisms of this effect were poorly understood. To the best of our knowledge, this is the first time that the expression levels of genes that are mainly involved in the conversion of monocytes macrophages to foam cells were evaluated after co-culture of platelets with monocytes. Our findings demonstrated that platelets increased the mRNA expression of CD36, ACAT, SRB, LXR-α, and ABCA1 genes. The role of these genes per se in the foam cell formation from monocytes is controversial. Multiple lines of evidence indicated that CD36 and ACAT pave the way for the formation of foam cells through enhancing the capability of the cells to uptake platelets/ox-LDL and cholesterol esterification, respectively [22, 23]. On the other hand, increased expression levels of SRB, LXR-α, and ABCA1 were also reported to be associated with the cholesterol
efflux from macrophage foam cells [24-26]. In consistent with our results, other studies showed that the gene expression of proteins such as CD36, ACAT1, LXR-α, and ABCA1 are increased during the macrophage-derived foam cell formation [22, 23, 31, 32]. Tsukamoto et al. also suggest a fundamental role for CD36 as well as SRB, but not SRA, in the formation of foam cells during OxLDL treatment of THP-1 cells [33]. Of particular interest, co-culture of monocytes with the platelets of CAD patients resulted in a superior up-regulation of CD36 and SRB mRNA expression, as compared with the results of co-culture using platelets of healthy volunteers.

5. Conclusion
Using an in vitro co-culture of monocytes and platelets, our study indicates that platelets from CAD patients could provoke monocyte differentiation into macrophages with a M2 phenotype, which in turn may participate in an atheroprotective process.

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Conflicts of interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

References
Li W, Katz BP, Spinola SM. Haemophilus ducreyi-induced IL-10 promotes a mixed M1 and M2 activation program in human macrophages. Infection and immunity 2012;IAI. 00912-12.


