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ОПРЕДЕЛЕНИЕ И ИМУНОФЕНОТИПИРОВАНИЕ ТРОМБОЦИТАРНО-МОНОЦИТАРНЫХ КОМПЛЕКСОВ В ПЕРИФЕРИЧЕСКОЙ КРОВИ С ПОМОЩЬЮ ПРОТОЧНОЙ ЦИТОФЛУОРОМЕТРИИ

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Резюме. Активированные тромбоциты образуют комплексы с циркулирующими лейкоцитами посредством мембраносвязанных молекул. Взаимодействие тромбоцитов с моноцитами лежит в основе патофизиологических механизмов, связывающих процессы тромбообразования и воспаления. Определение и анализ тромбоцитарно-моноцитарных комплексов (ТМК) позволяют выявить их физиологическую и патогенетическую роль, а также имеют важное диагностическое значение при изучении различных патологических состояний, в том числе акушерских. Цель исследования состояла в разработке метода определения количественного содержания ТМК в периферической крови, позволяющего сохранить основные фенотипические характеристики тромбоцитов и моноцитов и выявить их изменения при анализе биоматериала *ex vivo*. Предлагаемый метод сочетает в себе немедленную фиксацию образцов крови, иммуноцитохимическое флуоресцентное окрашивание дифференцировочных и активационных маркеров тромбоцитов и моноцитов с последующим лизисом эритроцитов и анализ с помощью проточной цитофлуориметрии. Было исследовано 14 образцов периферической крови, полученных от пациенток с отягощенным акушерским анамнезом в первом триместре текущей беременности. Показано, что процедура фиксации позволяет сохранить количественные и качественные характеристики ТМК, существующие *in vivo*, тогда как при отсутствии фиксации наблюдается многократное увеличение количества ТМК и уровня экспрессии активационных маркеров тромбоцитов и моноцитов *ex vivo*. Используемая панель моноклональных антител и примененные стратегии гейтирования обеспечивают оценку относительного и абсолютного количества ТМК и их фенотипических характеристик как в общей популяции ($CD45^+CD14^+$) моноцитов, так и в субпопуляциях «классических» ($CD14^+CD16^-$), «промежуточных» ($CD14^+CD16^+$) и «неклассических»

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($CD14^{low}CD16^{+}$) моноцитов. Выработанный подход позволяет оценить вклад отдельных субпопуляций моноцитов в формирование ТМК и степень их участия в физиологических и патофизиологических процессах. В отдельных образцах было выявлено увеличение количества ТМК, сопровождавшееся существенным повышением в них экспрессии активационного маркера тромбоцитов CD62P и снижением экспрессии его моноцитарного лиганда CD162. Данные перемены могут свидетельствовать об изменении активационного статуса комплексообразующих клеток и возможном участии ТМК в патофизиологических механизмах некоторых акушерских осложнений. Иммунофенотипирование ТМК позволяет дополнительно охарактеризовать их провоспалительный, прокоагулянтный и адгезионный потенциал и может быть применено как в фундаментальных исследованиях, так и в целях диагностики. В частности, данный метод может быть использован для определения и характеристики ТМК при акушерских осложнениях, сопровождающихся воспалением и нарушениями гемостаза.

Ключевые слова: тромбоцитарно-моноцитарные комплексы, тромбоциты, моноциты, периферическая кровь, иммунофенотипирование, проточная цитофлуориметрия

FLOW CYTOFLUORIMETRIC DETECTION AND IMMUNOPHENOTYPING OF PLATELET-MONOCYTE COMPLEXES IN PERIPHERAL BLOOD

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Abstract. Activated platelets aggregate with monocytes by their binding to membrane-bound molecules. Platelet-monocyte interaction is considered to underlie pathophysiological mechanisms bridging thrombosis and inflammation. Detection and analysis of platelet-monocyte complexes (PMC) provide means for revealing their physiological and pathogenetic roles and are instrumental in diagnostics of various pathological conditions, including obstetric complications. The aim of present study was to develop a technique for quantitative determination of PMCs in peripheral blood, that preserving phenotypic features of platelets and monocytes, and to reveal their changes by *ex vivo* analysis. The suggested procedure includes immediate fixation of blood sample, immunocytochemical staining with fluorochrome-conjugated specific antibodies against distinct activation and differentiation markers, followed by erythrocyte lysis, and flow cytometric analysis. We have analyzed fourteen samples of peripheral blood from the patients with a history of complicated pregnancies. The samples were taken in first trimester of the ongoing pregnancy. We have shown that quantitative and qualitative *in vivo* characteristics of PMC remained unchanged in pre-fixed samples, whereas the number of PMCs and expression levels of the platelet and monocyte activation markers dramatically increased in unfixed blood specimens. The set of monoclonal antibodies and gating strategies used in this study allows phenotyping and evaluation of percentage/absolute PMC counts in the total monocyte population ($CD45^{+}CD14^{+}$) and in the subpopulations of classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$), and non-classical ($CD14^{low}CD16^{+}$) monocytes. This approach provides insight into participation of different monocyte subsets in PMC formation, and their roles in physiological and pathophysiological processes. In some samples, elevated PMC proportion was observed, accompanied by significantly increased expression of CD62P platelet activation marker, and a decrease in its monocytic ligand CD162 expression. These changes suggested altered activation of PMC and their participation in pathophysiological mechanisms of some pregnancy complications. Immunophenotyping of PMC affords an opportunity to characterize their proinflammatory, procoagulant and adhesive properties; these results can be used for research and diagnostics. In particular, the method is suitable for detection and phenotyping of PMC in pregnancy complications and other pathological conditions associated with disorders of hemostasis and thrombosis.

Keywords: platelet-monocyte complexes, platelets, monocytes, peripheral blood, immunophenotyping, flow cytofluorimetry

Introduction

Circulating platelet-leukocyte complexes (PLC) come into focus of researchers in recent years. Activated platelets exhibit apparent capacity to form aggregates with leukocytes, in particular with monocytes and neutrophils [4]. Formation of PLC is predominantely mediated by the interaction of P-selectin (CD62P), expressed on the surface of activated platelets, with ligand PSGL-1 (CD162), which is constitutively expressed by leucocytes. The formed PLC are further stabilized by interaction between platelet membrane molecules CD40L, GPIbα, ICAM-2 and their leukocyte counterparts CD40, Mac-1 (CD11b/CD18), LFA-1, respectively [18].

Platelet-monocyte interaction is considered to be a pivotal event that underlies pathophysiological mechanisms bridging thrombosis and inflammation, in particular, proinflammatory effects, mediated by activated platelets [5]. It is accepted that platelet-monocyte complexes (PMC) are a more sensitive marker of *in vivo* platelet activation than commonly accepted parameter – CD62P expression level [15]. Quantitative detection of PMC as well as characterization of pro-inflammatory, pro-coagulant, and adhesive phenotypes are intended to reveal their physiological and pathophysiological roles. Moreover, this approach may be applied for diagnostic purposes.

Circulating monocytes are divided into three subsets by expression of surface markers CD14 and CD16 [19]. Determination and phenotyping of PMC in these subpopulations provide additional information about their activation and participation in the interaction with platelets.

Changes in the levels of total PMC and aggregates, formed by different monocyte subsets, were found in the circulation of patients with various inflammatory and thrombophilic disorders including acute myocardial infarction [12], cardiovascular diseases [2, 3], chronic obstructive pulmonary disease [2], antiphospholipid syndrome, systemic lupus erythematosus, and rheumatoid arthritis [10], tuberculosis [11]. Increased levels of PMC in pre-eclampsia suggest their pathogenetic role in this disorder [13]. We propose that other pregnancy complications, associated with prothrombotic state, are accompanied by activation of platelets and monocytes and manifest themselves as quantitative and qualitative changes in the characteristics of PMC.

Being engaged in the study of PMC roles in recurrent pregnancy loss, we faced with a choice of methodical approach. Currently, there is no conventional protocol of PMC determination in peripheral blood. Diversity of procedures employed by different research groups resulted in disparate data on the amount and phenotype of circulating PMC. Flow cytofluorimetry is widely used as an accessible and reliable analytical approach for identification and immunophenotyping of PMC. Methodical discrepancies mainly refer to sample preparation, selection of monoclonal antibodies/fluorochromes, and gating strategy. The most significant problem is platelet activation that results in *ex vivo* PMC formation. The phenomenon reflects neither physiological nor pathophysiological events and thus significantly interferes with determination of *in vivo* characteristics of PMC. It was demonstrated that spontaneous PMC formation began just after blood sampling and proceeded at high rate – 0.3-0.4% per minute. [8, 12]. In practice, it takes at least half an hour to proceed from blood sampling to analysis. Moreover, this interval may extend up to 1 h or more, if minimal transportation of the samples is needed. However, in the most existing protocols this point is overlooked, although PMC number may significantly increase during that time in comparison with their *in vivo* level.

The purpose of our study was development of the method of detection and quantification of PMC in peripheral blood samples, ensuring prevention of *ex vivo* platelet activation, preservation of phenotype characteristics of platelets, monocytes and PMC, and evaluation of their *in vivo* changes.

Materials and methods

Samples of peripheral blood were obtained from patients with history of pregnancy complications (recurrent pregnancy loss, non-developing pregnancy, intrauterine fetal death, antiphospholipid syndrome) in first trimester of ongoing pregnancy. Venous blood was sampled by venipuncture using 21-gauge needle and collected into vacutainer containing anticoagulant (3.8% sodium citrate). To avoid post-traumatic cell aggregation, first portion of blood (3-5 ml) was discarded. Since spontaneous platelet activation and formation of PLC occur *in vitro* in the absence of agonists, it is critical to fixate cells and aggregates as close as possible to the moment of blood sampling. Within 1-2 minutes after collection 0.5 ml blood was mixed with equal volume of CellFix (BD Biosciences).

After 1 hour 50 μl of fixed blood were immunolabeled with 5 μl of following fluorochrome-conjugated monoclonal antibodies: CD45-PerCP, CD14-Alexa Fluor®700, CD41a-APC, CD16-PE-Cy7™, CD62P-FITC, CD40-APC-H7, CD162-PE, CD142-PE, CD11b-PE (all BD Biosciences). To exclude non-specific binding and cell autofluorescence, isotype controls, conjugated with relevant fluorochromes, were used in addition to internal control, that provided optimal parameters for data acquisition. Samples were incubated for 20 min in dark at room temperature. Red blood cells were lysed by tenfold dilution of the samples with BD FACS™ Lysing Solution (BD Biosciences).

Samples were analyzed using FACSCanto II (Becton Dickinson) flow cytometer with FACSDiva software (BD Biosciences). Ten thousand events were collected in the platelet gate. For the analysis of monocytes and PMC, at least 5,000 events with relevant immunophenotype were collected in the monocyte gate. Leukocyte and platelet counts were determined with automated blood analyzer ADVIA 60 CT (Siemens). Statistical analyses were performed using Microsoft Office software package.

Results

Immediate and prolonged fixation of blood samples is a key issue of the suggested procedure. In preliminary experiments we compared PMC content and expression of activation markers of platelets and monocytes in unfixed blood and in the samples subjected to the immediate fixation. When compared to fixed blood, 2.5-4 fold increase in PMC percentage was observed after 1 hour without fixation, CD62P expression level in PMC increased twofold (from 9.7 to 18.7%), and CD11b demonstrated 8.5 fold increase, amounting up to 100%. Moreover, it was demonstrated that prolongation of fixation time up to 1 hour did not significantly affect such measured parameters as PMC amount and expression levels of platelet and monocyte markers.

We analyzed 14 peripheral blood samples obtained by the procedure described above.

Monocyte and platelets were determined with fluorochrome-conjugated monoclonal antibodies against specific antigenic markers.

Platelets were identified from the expression of CD41a, monocytes were recognized from the expression of both CD45 and CD14. Platelet-monocyte aggregates were recognized as CD14 and CD41a double positive events. Monocyte subsets were determined from differential CD14 and CD 16 expression.

Gating strategy used for determination of unaggregated platelets and representative histogram, demonstrating CD62P expression in this population, are shown in Figure 1. P-selectin (CD62P) is a marker of platelet activation, and levels of CD62P expression in free platelets may be compared to the levels of CD62P expressed by platelets, aggregated with monocytes (PMC). Median percentage of $CD62P^+$ free platelets in the analyzed samples was 2,7% (0,1-4,6%).

Gating strategy for detection of free monocytes and PMC is shown in Figure 2. This approach ensures quantification of PMC and determination of surface antigenic markers in total monocyte population and monocyte subsets: classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$), and non-classical ($CD14^{low}CD16^+$). In the analyzed samples, median percentage of PMC in the total monocyte population was

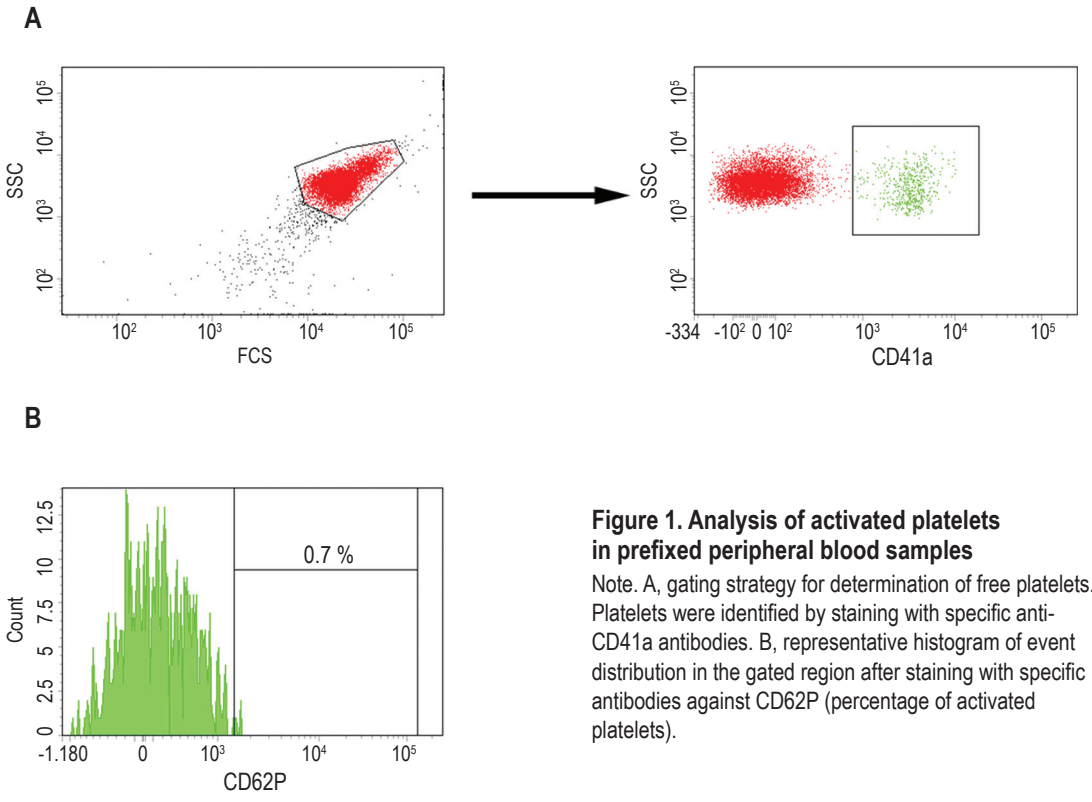

Figure 1. Analysis of activated platelets in prefixed peripheral blood samples

Note. A, gating strategy for determination of free platelets. Platelets were identified by staining with specific anti-CD41a antibodies. B, representative histogram of event distribution in the gated region after staining with specific antibodies against CD62P (percentage of activated platelets).

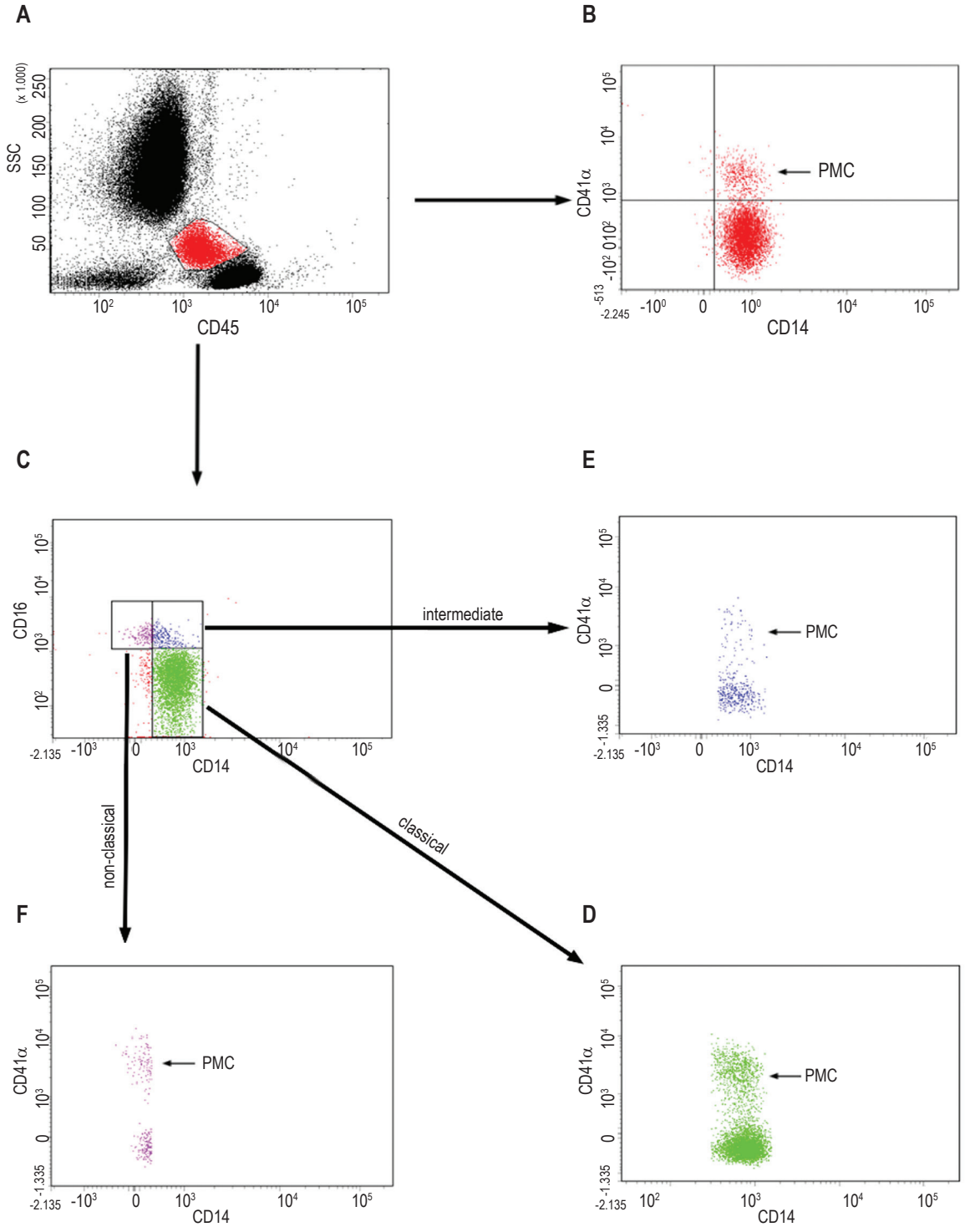

Figure 2. Gating strategies for the analysis of PMC in the total monocyte population (upper panel) and monocyte subsets (middle and lower panel)

Note. On SSC/CD45 dot plot the region, corresponding to morphological and phenotypic characteristics of monocytes, was set (A). In the total monocyte population, PMC were identified by co-staining with anti-CD14 and anti-CD41a antibodies (B). Monocyte subsets identified with anti-CD14 and anti-CD16 antibodies (C), were classified as classical ($CD14^{+}CD16^{-}$) (D), intermediate ($CD14^{+}CD16^{+}$) (E), and non-classical ($CD14^{low}CD16^{+}$) (F). In the monocyte subsets, PMC were identified as CD41a-positive events.

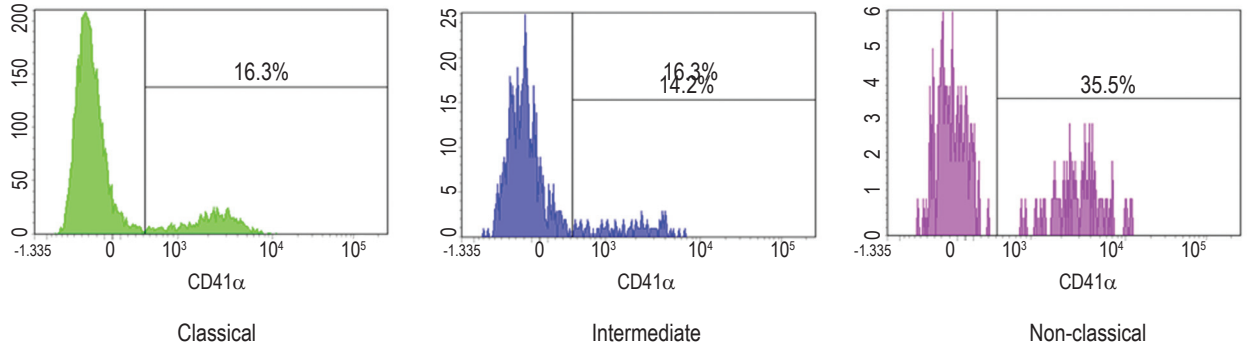

Figure 3. Representative histograms of PMC distribution in the monocyte subsets
Note. Percentages of PMC in the monocyte subsets are shown on the figures.

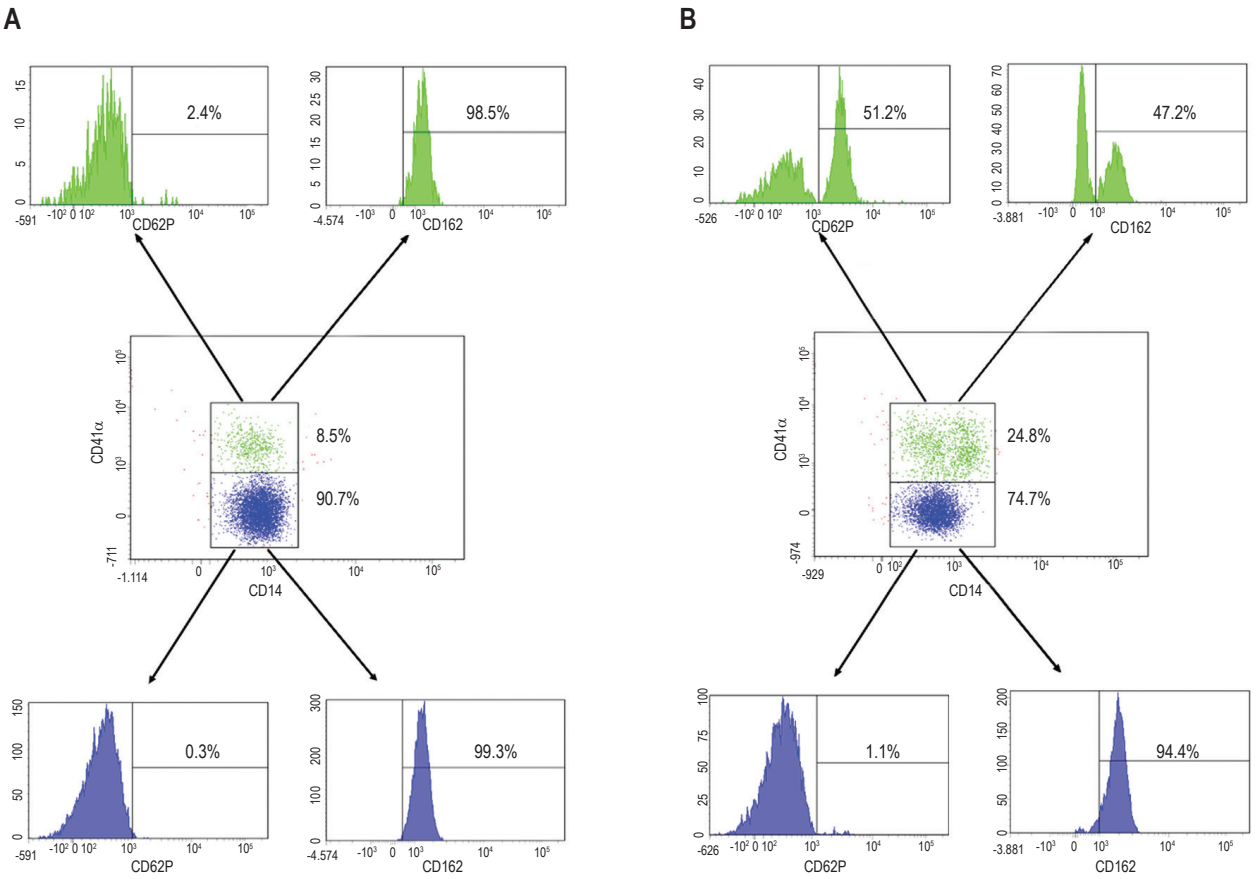

Figure 4. Analysis of PMC formation and expression of surface markers of platelets and monocytes in different samples of prefixed peripheral blood
Note. The marker of platelet activation CD62P (P-selectin) and its ligand CD162 (PGSL-1) were determined on the surface of PMC and free monocytes by staining with corresponding specific antibodies. A, monocyte marker CD162 was expressed by both PMC and free monocytes, whereas platelet marker CD62 was detected in less than 5% PMC. B, PMC, but not free monocytes, demonstrated 50% increase in CD62P expression accompanied by comparable decrease in CD162 level.

12.5% (5.5-16.7%). Representative histograms demonstrate potential of the method to quantify the differences between monocyte subsets in their ability to form complexes with platelets: $CD14^{+}CD16^{-}CD41\alpha^{+}$, $CD14^{low}CD16^{+}CD41\alpha^{+}$, and $CD14^{+}CD16^{+}CD41\alpha^{+}$. Figure 3 demonstrates that in the sample, non-classical ($CD14^{low}CD16^{+}$) monocytes formed more than twice as many aggregates with platelets as two other

monocyte subsets (classical and intermediate) and total monocyte population.

The suggested method is not only applicable for detection of PMC in total monocyte population and monocyte subsets, but for quantitative characterization of platelet-monocyte interaction in PMC by measuring expression of relevant molecules. Binding of platelet P-selectin (CD62P) to CD162 on the monocyte surface plays a key role in PMC formation. In the majority of peripheral blood samples, analyzed in our study, CD62P expression levels did not exceed 5%, whereas all free monocytes and PMCs were CD162-positive (Figure 4A). However, in two samples significant increase in CD62P expression and decrease in CD162 expression by ~50% was observed (Figure 4B). Noteworthy is that these changes were observed in PMCs ($CD14^+CD41\alpha^+$), but not in free monocytes ($CD14^+CD41\alpha^-$). Moreover, percentage of PMC increased, and percentage of free monocytes decreased (Figure 4B). No correlation was revealed between this phenomenon and clinical findings of the patients.

In the analyzed samples, no significant CD40 and CD142 expression was determined. CD11b expression level varied from 1.2 to 35.1% with median value of 14%.

Percentage values obtained with flow cytometry can be converted into absolute values by using data obtained with blood analyzer. Thus, concentrations of PMC and free cells with specific phenotypes can be calculated.

Discussion

Based on earlier described procedures assuming pre-fixation of blood samples [2, 6, 12], we developed the method of quantifying and phenotypical characterization of PMC in the peripheral blood that can be used for studying the role of platelet-monocyte interactions in the pathogenesis of pregnancy complications.

We used sodium citrate as anticoagulant since it was demonstrated to have a minimal effect on PMC formation [8]. In our preliminary experiments with different anticoagulants we came to the same conclusion. Sodium citrate is anticoagulant of choice in many studies on PMC detection and characterization [2, 7, 11, 12, 16].

In platelet research, special requirements are set in sampling and sample preparation. The procedures should be performed in such a way as to minimize *ex vivo* platelet activation. On the other hand, immunocytochemical staining implies best preservation of native conformation and expression density of the antigenic determinants, recognized by monoclonal antibodies. Mild fixation of biomaterial, containing the cells of interest, meets the requirements. It is achieved by applying fixing agent (paraformaldehyde) at lower concentrations than those used in the standard protocols. The procedure of immediate fixation, used in our study, provided preservation of antigenic phenotype from blood sampling to laboratory analysis.

The described method excludes undesirable sample handling including sedimentation by centrifugation, cell separation in density gradient, washing with buffer solutions. Thus, immunocytochemical staining with fluorochrome-conjugated monoclonal antibodies was carried out directly in the fixed blood sample.

The panel of specific antibodies and conjugated fluorochromes was set for differential counting of PMC and free platelets and monocytes as well as for effective determination of the levels of expression of inducible antigenic markers on the surface of the cells and aggregates.

The results of the study demonstrate that the suggested method offers a means for a reliable identification of PMC and determination of their activation state in total monocyte population and in the monocyte subsets.

Increased amount of PMC, detected in some samples, accompanied by increased expression of platelet activation marker CD62P and decreased expression of its ligand CD162, suggest changes of activation state of PMC and their roles in pathophysiological mechanisms of some pregnancy complications. To reveal correlation between changes in these parameters and clinical signs of certain pregnancy complications, it is necessary to analyze a considerable amount of blood samples obtained from patients with physiological pregnancy and complicated pregnancies.

Likewise, shift in balance of PMC formed by different monocyte subsets apparently reflects the different roles of these subpopulations in physiological and pathological processes. It is evident that PMC and phenotypic markers of activation should be determined not only in total monocyte population, but also in the monocyte subsets since the alterations occurring in the minor subpopulation may be undetectable in the whole population.

In our study, we used specific antibodies against the surface antigens, that are the hallmarks of proinflammatory and procoagulant monocyte phenotype. CD40L receptor CD40 mediates platelet-monocyte interaction in inflammation and thrombosis [9]. Tissue factor (CD142) plays a key role in the initiation of blood coagulation [17]. Although we did not find significant expression of these factors in the analyzed samples, we believe that determination of CD40 and CD142 should be included in the analysis of PMC in patients with thrombophilic state. Detection of CD11b seems to be useful to characterize adhesive phenotype of free monocytes and PMC. This molecule is a part of leukocyte integrin Mac-1 (CR3, CD18/CD11b)

and mediates various monocyte functions including PMC stabilization by interacting with platelet glycoprotein GPIb [14], binding to complement components, fibrinogen, and ICAM-1 molecules expressed on the surface of endothelial cells.

Conclusion

We developed accessible and reproducible method, that provides preservation of phenotypic characteristics of PMC and ensures delayed analysis of blood samples, quantifying of PMC, and determination of activation state. The suggested procedure includes immediate mild fixation, minimal sample handling, optimal gating strategies, and relevant panel of specific antibodies for identification of the differentiation and activation antigenic markers using flow cytofluorimetry. We believe that the suggested method can be used for detection and characterization of platelet-monocyte complexes in patients with various pathologies accompanied by inflammation and thrombosis, including pregnancy complications.

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