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Enumeration of circulating fibrocytes for clinical use in asthma by an optimized single-platform flow cytometry assay

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ABSTRACT

Background: Elevated numbers of circulating fibrocytes are associated with inadequately controlled asthma, poor response to available therapies, and increased risk of adverse outcomes. The lack of reliable and clinically-applicable assays precludes a proper evaluation of blood fibrocyte count as a prognostic biomarker in asthma. This report concerns the use of a multiparameter flow cytometry assay for the enumeration of fibrocytes in the whole blood.

Methods: Consenting fibrocyte donors were 19 patients with asthma well controlled by current treatment, 16 patients with treatment-resistant asthma, 9 patients with transiently uncontrolled asthma and 14 age-matched normal individuals. Blood sampling was performed once in patients with transiently uncontrolled asthma and twice, at an interval of one week, in the other subjects. The assay was performed in 100 μl of whole blood and involved a sequential gating strategy and absolute fibrocyte counting with a single instrument (single-platform assay).

Results: The quantification of circulating fibrocytes by this assay was analytically and clinically valid. In individuals with stable clinical conditions, the repeatability of blood fibrocyte counts over one week was good. The intraclass correlation coefficient was 0.939 and 96.88% of the total variability reflected on-average differences among the tested subjects. Stabilized blood samples could be stored at 4 °C for up to 96 h before processing.

Conclusions: The novel assay for the enumeration of fibrocytes in the whole blood is reliable and clinically applicable.

General significance: This report demonstrates the validity and reliability of the first optimized assay for the enumeration of circulating fibrocytes in multicenter clinical trials.

1. Introduction

Asthma is a common inflammatory disorder of the airway for which there is no cure [1]. Optimal asthma management consists in the achievement and maintenance of the best possible clinical control and in the prevention of disease exacerbations, which are most frequently triggered by allergen exposure and viral infections and increase the risk of future adverse outcomes [1,2]. Clinical parameters of asthma control include the frequency of symptoms during the day and at night or on awakening, presence of activity limitation because of symptoms, and level of airflow obstruction [1]. The lack of clinical control of asthma reflects ongoing airway inflammation and indicates inadequate anti-inflammatory treatment [1,2]. When patients with asthma responsive to the currently available anti-inflammatory therapies suffer from an acute exacerbation of their disease, asthma control can be achieved again by increasing the intensity of the anti-inflammatory treatment. In contrast, patients with treatment-resistant disease have frequent signs and symptoms of airflow obstruction, severe exacerbations requiring hospitalization, and persistent airway inflammation not responding to available therapies. Ongoing airway inflammation is associated with an increased risk of adverse outcomes because the chronic

Abbreviations: 7-AAD, 7-amino-actinomycin-D; AF, Alexa Fluor; α-SMA, α-smooth muscle actin; BMC, blood mononuclear cell; CCC, concordance correlation coefficient; CI, confidence interval; COL1, type I collagen; C_T, threshold cycle; ET-1, endothelin-1; FSC, forward scatter; ICC, intraclass correlation coefficient; MFI, mean fluorescence intensity; PB, Pacific Blue; SD, standard deviation; SS, sum of squares; SSC, side scatter

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inflammatory process promotes the development of irreversible structural changes in the bronchial tubes that lead to fixed airway narrowing and progressive loss of lung function [1,2]. Easily accessible biomarkers of airway inflammation and asthma control are therefore needed for monitoring the effects of available treatments in patients at increased risks of adverse outcomes and to assess their responsiveness to novel therapeutic candidates in clinical trials [2–4]. Blood fibrocyte count is emerging as one of these easily accessible biomarkers.

The fibrocytes are circulating $CD45^+CD34^+HLA\text{-}DR^+CD11b^+CD13^+CD16^-CD115^-$ cells that exhibit both hematopoietic and stromal-like features and possess antigen-presenting activity [5–9]. They produce several cytokines and growth factors and express type I collagen (COL1) genes and protein [5,7,10,11]. Their proinflammatory and profibrotic properties [6,9–11] are relevant to asthma [9,12–15], and there is mounting evidence [12–18] that these cells substantially contribute to the progression of the structural changes that ultimately lead to fixed airway narrowing in patients with frequent exacerbations and treatment-resistant disease. Elevated numbers of fibrocytes are present in the circulation and in the airways of these patients [10,14,17,18] as a result of the release of ligands of the C – C chemokine receptor 5 and other cytokines that are known to mediate the output of fibrocytes from the bone marrow and their recruitment to the inflamed tissue [18,19]. Moreover, the presence of an elevated number of fibrocytes in the peripheral blood of asthmatic individuals with inadequately controlled disease not only reflects ongoing airway inflammation [10,14,18] but is also associated with persistent airflow obstruction and accelerated reduction of lung function over time [17]. Thus, elevated blood fibrocyte counts in asthma are indicative of inflammatory and structural alterations that can otherwise be detected in a non-invasive way solely by combining cellular analysis of induced sputum with high-resolution computed tomography [20]. The use of blood fibrocyte count as an asthma outcome may also be advantageous in comparison with other prognostic biomarkers because it does not seem to be affected by treatment with corticosteroids. In fact, patients with treatment-refractory asthma by definition use the highest possible dose of inhaled corticosteroids, often in combination with oral corticosteroids, and yet they have substantially higher numbers of fibrocytes in their peripheral blood and airways than patients with treatment-responsive asthma of any severity [14,18].

Unfortunately, the evaluation of the validity and utility of blood fibrocyte count as a novel cellular biomarker in asthma is currently precluded by the absence of reliable and clinically-applicable analytical assays. The experiments described in the present report were therefore conducted to evaluate the clinical applicability of an optimized multiparameter flow cytometry assay for the enumeration of fibrocytes in the whole blood, which involves a sequential gating strategy and direct absolute fibrocyte counting with a single instrument by using the single-platform technology [21–23].

2. Materials and methods

2.1. Patients

The experiments were conducted during the course of two recently published studies [16,18], according to protocols approved by the appropriate review board, and the results are cumulatively reported here. Consenting fibrocyte donors were 19 patients with asthma well controlled by current treatment [18], 16 patients with treatment-resistant asthma [18], 9 patients with transiently uncontrolled asthma because of an allergen-induced exacerbation [16], and 14 age-matched normal individuals [16]. The demographic and clinical characteristics of these patients are described in every detail in the two published articles [16,18]. Blood sampling was performed once in patients with transiently uncontrolled asthma and twice, at an interval of one week, in the other subjects.

2.2. Enumeration of circulating fibrocytes

Blood specimens were collected by venipuncture into evacuated tubes containing tripotassium ethylenediamine tetraacetate anticoagulant. Aliquots of the blood samples were either left untreated, and processed within 4 h, or mixed with the cellular surface antigen-stabilizing agent TransFix (200 μl per 1000 μl blood, Life Technologies, Carlsbad, CA, USA) [24,25] and stored at 4 °C for up to 96 h before processing.

One hundred μl of treated or untreated blood were added to a 5-ml round-bottom tube containing 2 μl of the viability dye 7-amino-actinomycin-D (7-AAD, BD Biosciences, San Jose, CA, USA), which is detected on the PerCP-Cy5.5 channel, and appropriate dilutions in stain buffer (BD Biosciences) of the following fluorochrome-labeled monoclonal antibodies (all mouse $IgG_{1\kappa}$): 2.5 μl CD3-PerCP-Cy5.5, 2.5 μl CD19-PerCP-Cy5.5, 2.5 μl CD20-PerCP-Cy5.5, 2.5 μl CRTH2/CD294-PerCP-Cy5.5, 5 μl CD45-AmCyan, 5 μl CD34-PE, 5 μl CD11b-Pacific Blue (PB), 10 μl CD16-FITC (BD Biosciences), and 5 μl CD115-APC (R&D Systems Europe, Abingdon, United Kingdom). After incubation for 20 min at room temperature in the dark, erythrocytes were lysed with 2 ml fixative-free High-Yield Lyse solution (Life Technologies). CountBright absolute counting beads (Life Technologies) were added to allow an absolute count of the target cell population according to the manufacturer's instruction, and samples were analyzed immediately without any wash steps.

The flow cytometer was a 4-laser BD SLRII (BD Biosciences) and the instrument was set up using BD Cytometer Setup and Tracking beads. Data were acquired and analyzed with the BD FACSDiva software (BD Biosciences). Compensation settings were performed with a set of compensation tubes using the BD CompBead antibody-capturing particles and the compensation setup tool in the BD FACSDiva software. Antibodies were titered for no-wash staining. Fluorescence-minus-one controls were used for accurate definition of cells that had fluorescence above the background levels [26].

The gating strategy applied for the enumeration of fibrocytes is shown in Fig. 1. Because circulating fibrocytes are present in the $CD45^+$ blood mononuclear cell (BMC) fraction [5–7], this population was initially gated on the basis of the side scatter (SSC) characteristics and CD45 expression, and debris and platelets were eliminated. The beads were gated for counting and excluded from cell analysis. A dump channel was then established on the PerCP-Cy5.5 channel to exclude $7\text{-}ADD^+$ non-viable cells and some of the unwanted cells that express CD11b, comprising CD3-positive T lymphocytes, $CD19^+CD20^+$ B lymphocytes and B blasts, CRTH2-positive immature and contaminating mature eosinophils, and $CRTH2^+$ basophils [21,27]. $CD45^+$[7-ADD/CD3/CD19/CD20/CRTH2]$^-$ cells were gated in the plot PerCP-Cy5.5 *versus* forward scatter (FSC). Contaminating $CD16^+CD11b^+$ neutrophils, $CD115^+CD16^+CD11b^+$ monocytes and $CD115^+$ $CD16^-CD11b^+$ mature and immature monocytes [21,28] were than sequentially excluded from this population by gating CD16- and CD115-negative cells in the plot CD16-FITC *versus* SSC and CD15-APC *versus* SSC, respectively. Fibrocytes were identified within the $CD45^+$[7-ADD/CD3/CD19/CD20/CRTH2]$^-CD16^-CD115^-$ population on the basis of the coexpression of CD34 and CD11b, which distinguished these cells from the other remaining $CD45^+$[7-ADD/CD3/CD19/CD20/CRTH2]$^-CD16^-CD115^-$ cells, including $CD45^{dim}CD34^+CD11b^-$ blasts/progenitors [29], circulating $CD45^+CD11b^-CD34^-$ myeloid and plasmacytoid dendritic cells [9,30], and $CD45^+CD34^-$ $CD11b^+CD16^-CD56^+$ NK cells [21,27]. In the CD45/SSC plot, the gated $CD45^+$[CD3/CD19/CD20/CRTH2]$^-CD16^-CD115^-CD11b^+CD34^+$ cells showed an SSC slightly higher than that of lymphocytes and similar to that of dendritic cells [27] and some myeloid progenitors [29]. At least 10^5 cell events were acquired for each assay before cell analysis and absolute cell counting. The intra-assay variability was assessed by analysis of the absolute fibrocyte counts in three separate 100-μl aliquots of each blood sample. The coefficient of variation ranged from 2.84 to 5.73% in fresh blood samples, from 4.15 to 8.28% in blood samples