Circulating Fibrocyte Level in Children with Cystic Fibrosis

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Research Article

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Abstract

This study aimed to evaluate circulating fibrocyte levels of cystic fibrosis (CF) patients at stable and exacerbation period. The study group consisted of 39 patients diagnosed with CF and 20 healthy controls. Individuals included in the study were divided into 3 groups as CF, CF exacerbated and healthy control groups and their circulating fibrocyte levels were compared. Findings from pulmonary function test (PFT) and high-resolution computed tomography (HRCT) of the lung were evaluated and compared with each other.

The circulating fibrocyte count was found significantly higher in patients with CF compared to the exacerbated and control groups. No correlation was found between the forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) values in PFT and the circulating fibrocyte count. The circulating fibrocyte count in patients (in CF group) with positive findings in HRCT was statistically significantly lower.

Conclusion: The circulating fibrocyte level in the peripheral blood of the patients with CF was increased.

What Is Known

- Chronic inflammation is responsible for lung damage in patients with cystic fibrosis.

What Is New

- Fibrocytes might play a role in the pathophysiology of CF lung damage.

Introduction

Cystic fibrosis (CF) occurs as a result of the defect in chloride channel called cystic fibrosis transmembrane regulator (CFTR) protein in epithelial cell membrane [1,2]. Structural and functional defects in CFTR protein lead to the disruption of ion transport in epithelium cell plasma membrane of organs such as lung, pancreas, liver, intestines, sweat glands and epididymis [3-4]. Although lung damage occurs in CF due to chronic endobronchial infection, neutrophil predominant inflammation has been shown to initiate in asymptomatic and culture-negative infants. Structural lung disease develops in CF at the age of 5 due to infection and chronic inflammation[5,6]. In studies conducted on pigs with neonatal CF, structural defects in the lungs at birth have been shown and it has been reported that lung damage due to CFTR starts in the fetal period without infection and inflammation [7].

Circulating fibrocytes are fusiform and bone morrow-derived mesenchymal progenitor cells that were first described in 1994. It was revealed that circulating fibrocyte levels in peripheral blood and affected tissue increased in diseases accompanied by fibrosis [8]. The circulating fibrocytes migrate into inflamed or injured tissues and transform into fusiform fibroblast-like cells that are classified as mature fibrocytes. They constitute 0.1-0.5% of non-erythrocyte cells in extracellular matrix [9]. They express hemopoietic

surface markers such as CD34, leukocyte surface markers such as CD45 and specific markers such as collagen 1 and alpha-smooth muscle actin (a-SMA) [10]. In the context of normal wound healing and fibrotic progression, circulating fibrocytes may selectively migrate into wound sites and differentiate into fibroblast-like cells within extracellular matrix accumulation areas [11,12]. Recent studies showed that in various type of lung diseases (such asbronchopulmonary dysplasia (BPD), idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), romatoid arthritis- interstitial lung disease (RA-ILD) and systemic sclerosis (SSc)-associated interstitial lung disease)fibrocyte levels are increased both in blood and lung tissue [8,13-17]. More recently increased fibrocyte accumulation has beenshown in the lung tissue of CF patients with end stage lung disease [18].These data suggested us circulating fibrocytes might have potential role in CF lung disease.

Based on this information, this study aimed to evaluate the level of circulating fibrocyte in CF patients and the relationship between imaging findings of lung disease, pulmonary function tests (PFT) and pulmonary exacerbation of the patients with CF and circulating fibrocyte level in the peripheral blood of these patients.

Method

This study was performed prospectively at Erciyes University, Faculty of Medicine, between May 2018 and June 2019. The Ethical approval was obtained from the University of Erciyes (2018/207). The study was funded by the Scientific Research Projects Funding of Erciyes University (project no: TTU-2018-8356). Written and oral consents were obtained from the parents after they were informed about the procedures and objective of the study.

The diagnosis of CF is based on sweat chloride levels (> 60 mmol/L), identification ofpathogenic mutation on both CF alleles and characteristic symptoms of CF [4,19]. Patients aged 3-18 years were included in the study. Follow-ups and treatments of the patients were evaluated by the clinicians. Blood samples were collected from the healthy controls and CF patients. Healthy control group were selected from those who were between 3-18 years old, had normal systemic examinations, had no upper respiratory tract infection in the last month, had no additional systemic disease, and were admitted to the hospital for control in the well-child outpatient clinic.Blood samples of CF patients were taken when there were no active symptoms at the same time with PFT and HRCT examination. All CF patients followed up for 1 year. Patients with these symptoms were considered pulmonary exacerbations:1) new crackles, 2) increased cough, 3) increased sputum, and 4) a relative decline of more than 45% in weight-for-age percentile, 5) new and increased hemoptysis, 6) high fever, 7) At least 10% reduction in pulmonary functions, 8)new radiological findings suggestive of lung infection [20,21]. Eight of 39 CF patients with these symptoms admitted to the hospital were accepted pulmonary exacerbation and at the beginning of the pulmonary exacerbation blood samples were taken again.

Other examination results apart from circulating fibrocyte levels were obtained from the Information Management System of the hospital.

SFT was applied by the same person to the patients diagnosed with CF with Jaeger MasterScope Body. The device was calibrated every day before starting the tests. Before the test, the patients were informed about the techniques to be performed.PFT was not applied to children under six years of age and non-cooperative children. It was applied to over six years of age and cooperative children.Patients with FEV1> 80 were considered normal and FEV1 <80 were considered decreased. Patients with FVC> 80 were considered normal and FVC <80 were considered decreased.

High-resolution CT of the thorax was performed on patients in the routine examination of our centerwith Toshiba Aqullion CT scanner and the results were evaluated by the same person.HRCT findings of the patients were scored according to the scoring system based on the study by Bhalla et al. (Table 1) [22]. The patient's total CT points were calculated on these indicated morphological findings in the table 1, with the highest possible point being 25. HRCT scores of the patients were graded as in the Shwachman–Kulczycki system. The total CT point was subtracted from 25, in order to find the HRCT score in the Shwachman–Kulczycki scoring system. According to The Shwachman - Kulczycki system, 21-25 points excellent, 16-20 points well, 11-15 points mildly affected, 6-10 points moderately affected and 0-5 points severe affected, were considered [23].

Determination of Circulating Fibrocytes

For the analysis of circulating fibrocytes, 3 ml of blood was put into a single EDTA tube. For the isolation of peripheral blood mononuclear cell (PBMC), Ficoll-Hypaque (GE17-1440-03) density gradient was used on fresh whole blood samples according to the instructions of manufacturer. After the venous blood was diluted by 1:1 with phosphate-buffered saline (PBS), 3 mL of diluted blood was transferred over Ficoll and spun at 1,500rpm for 30 minutes at room temperature while the break was switched off. PBMCs were collected from the interface and the cells were counted. They were stored at -80 degrees in patient's own serum including 10% dimethyl sulfoxide(DMSO) until the day they would be used. When the cells were to be used water bath was maintained at 37^{o}C and the cells in cryovial tube were thawed quickly in water bath, transferred into 15 mL tubes with complete medium and centrifuged at 1,500rpm for 5 minutes. The supernatant was removed and the cells were used in the experiment. For each sample, 1-3x10^{6} cells were collected. For wash steps, 2 ml of flow cytometry staining buffer (FACS buffer) was used. After centrifugation, the supernatant was removed and the cells were resuspended. Then, 100 µl of FACS buffer including 0.5–1 µg of PerCP/Cy5.5 anti-human CD45 antibody was added. The samples were vortexed and incubated at +4 degrees in the dark for 30 minutes. The cells were washed with 2 ml of FACS buffer. After centrifugation, the supernatant was removed and the cells were washed again. After centrifugation, the supernatant was aspirated. After 100 µl of fixation buffer was added the cells were fixed and cell pellet was resuspended. They were incubated at room temperature for 20 minutes. Then, 1 ml of permeabilization buffer was added into each tube. After centrifugation, the supernatant was removed. The cells were resuspended and incubated at room temperature with 100 µl of permeabilization buffer for 5 minutes. They were washed with 2 ml of permeabilization buffer and centrifuged and the supernatant was removed. The cells were resuspended with 100 µl of permeabilization buffer including 0.5 – 1 µg of collagen type 1 antibody or isotype control, vortexed and incubated at +4 degrees in the dark for 30

minutes. The cells were washed with 2 ml of permeabilization buffer and centrifuged and the supernatant was removed. The cells were resuspended. Then, 100 µl of permeabilization buffer including 0.5 – 1 µg of collagen secondary antibody was added. The cells were vortexed and incubated at +4 degrees in the dark for 30 minutes. The cells were washed with 2 ml of FACS buffer and centrifuged and the supernatant was aspirated. They were resuspended with 200 µl of FACS buffer and their protein expression levels were measured in flow cytometry.

Statistical Analyses

Data obtained were statistically analyzed on Turcosa, a cloud-based statistical analysis system (TURCOSA A.S. Ltd. Sti. Kayseri, Turkey). Firstly, descriptive statistics were performed with the data obtained. Then, Shapiro-Wilk test was used to test whether the variables were normally distributed or not. Homogeneity of variance of the variables was analyzed with Levene's test. Kruskal-Wallis test, Student t test and Mann-Whitney U test were used to test the changes of quantitative variables among categorical groups by considering whether the variables were normally distributed or not. Pearson's correlation coefficient was used to evaluate the relationship of quantitative variables with each other. Fisher's Exact test and Chi-square analysis were used in the analysis of qualitative variables among categorical groups. The significance level was accepted as "$p<0.05$".

Results

There were 39 patients in CF group, and 20 patients in the control group.There was no significant difference between CF and healthy control group with respect to age and gender. Demographic information of the patients is shown in Table 2.

Flow cytometry was used to determine the percentage of circulating fibrocytes. The cells weregated so that the gate includes lymphocytes, monocytes and some leftover granulocytes (post-freeze-thaw) according to FSC-A (Forward Scatter) and SSC-A (Side Scatter) properties. Then, FSC-H and FSC-A gating was performed to gate on singlet cells and another gating was performed to gate on the cells expressing CD45. The last gating was performed to determine the amount of collagen 1 produced by CD45 positive cells,representative flow plots in Figure 1Ashows thegating strategy from the beginning to the last stage.

The circulating fibrocyte absolute numbers per ml blood and theirpercentagesamong CD45+ cells of paired CF patients during exacerbation period and before were given in Figure 1B. Both the absolute number (per mL blood) and the percentages(among CD45+ cells) of circulating fibrocytes were significantly higher in the stable CF groupcompared to the CF exacerbation patients. Next the analysis was performed with groups including the paired and unpaired patients in the stable and exacerbation groups. Similarly, both the absolute number and the percentages of circulating fibrocytes of patients in the CF group was significantly higher compared to healthy control group as well as CF exacerbation group when all patients were included, supporting the paired analysis (Figure 2, Table 2).

All CF patients were followed up for a period of one year and pulmonary exacerbation occurred in eight of them. Thus, 8 of 39 patients were examined longitudinally before and during pulmonary exacerbation. In these eight patients circulating fibrocyte levels were significantly decreased when compared with their stable period (Table 2).

14 patients could not perform PFT due to the lack of cooperation. 25 patients could perform PFT. The FEV1 value were <80% in 8 patients. The median circulating fibrocyte value was 64195 (8900-648492) in lower FEV1 patients. The FEV1 value were >%80 in 17 patient. The median circulating fibrocyte value was 399818 (7490-7796490) in normal FEV1 patients. The circulating fibrocyte values were statistically decreased in patients with lower FEV1 values (Table 3). The FVC value was<80% in 6 patients and FVC value were >%80 in 19 patients. The median circulating fibrocyte value was 86103 (8900-648492) and 337176 (7490-7796490) respectivelyAlthoughthe circulating fibrocyte values were lower in decreased FVC group. but there was no significant difference when compare with normal FVC group. (Table 3).

In PFT, median FEV_1 value was 55% (22-133), median FVC value was 56% (27-123) and median circulating fibrocyte value was 725141 (7490-7796490) in patients with CF. No correlation was found between the circulating fibrocyte counts and FEV_1 percentage in PFT of patients with CF. No correlation was found between the circulating fibrocyte counts and FVC value in PFT of patients with CF. Circulating fibrocyte levels were significantly decreased in patients with low FEV_1 and FVC when compared with CF patients with normal PFT (Table 3).

Based on HRCT finding CF patients divided into 2 groups according to the Shwachman - Kulczycki system scores.Group 1 patients (n:19) score was 0-15 (mildly affected, moderately affected and severe).Group 2 patients (n:20) score was 16-25 (good and excellent). The circulating fibrocyte count of the patients in the group 1 was statistically lower when compared with group 2 patients (Table 3).

Discussion

In this study, the circulating fibrocyte counts were significantly higher in patients with CF compared to the control group. To the best of our knowledge there is no study analyzing the circulating fibrocyte level in patients with CF.However, there are studies on circulating fibrocyte levels in different diseases accompanied by pulmonary damage in the literature. In chronic lung diseases such as BPD, COPD, RA-ILD, IPF and systemic sclerosis (SSc)-associated interstitial lung disease circulating fibrocytes levels were higher both in the blood and BAL of the patients. [8, 13-17]. The authors argued that fibroblast transformation from mesenchymal progenitor cells increased secondary to chronic inflammation and that elastin and collagen secreted from fibroblasts were responsible for pulmonary damage in BPD [8].These findings are consistent with the notion that circulating fibrocyte count increased in diseases which were accompanied by chronic inflammation and which caused pulmonary damage. These results are consistent with our finding that circulating fibrocyte levels were higher in the patients with CF accompanied by chronic inflammation. Recently Kasam et al [18] showed that fibrocytes are increased in the lung tissue of six CF patients with end stage lung disease whounderwent lung transplantation.In the

same study they found fibrocytes in the BAL fluid of younger CF patients which is normally undetectable in healthy humans. Our findings suggest that circulating fibrocytes are one of the source of increased fibrocytes in the lung tissue and BAL of the CF patients. The findings of both studies suggest that not only resident fibroblasts, but also circulating fibrocytes which migrate to the lung tissue due to the prolonged damage and inflammation might have potential role in the tissue repair and/or tissue damage of CF patients.

Interestingly, during the exacerbationperiod of patients with CF, circulating fibrocyte levels were decreased when compared with the stable period.The lack of increase in fibrocyte levels during acute pulmonary exacerbation is an issue that needs to be explained. In the study on patients with RA, circulating fibrocyte levels did not rise during RA activation [16]. Similarly, Borie et al. [17] found in their study that circulating fibrocyte counts did not increase in patients with acute exacerbation of IPF. On the contrary, Moeller et al. [13] found that circulating fibrocyte count in the peripheral blood increased during acute exacerbation of patients with IPF and the reasons for divergent results from these two studies are unknown. Nevertheless, data indicate that circulating fibrocytes appear as effector cells in chronic inflammation and that they play arole in the pathogenesis of chronic inflammatory condition [11]. One plausible explanation as to why circulating fibrocyte count did not increase in the peripheral bloodduring the exacerbationperiod of the patients with CF may be that during exacerbation, the fibrocytes in the peripheral blood might have already migrated into the tissues and thus was observed less in the peripheral blood.

Interestingly the circulating fibrocyte levels were higher in CF patients with less affected HRCT findings and had normal PFT. CF patients with lung damage on HRCT and decreased PFT had higher circulating fibrocyte levels compared to the healthy control subjects. We could not find any correlation between fibrocyte levels and FEV1 and FVC. There wasn't any correlation between the circulating fibrocyte count and the findings in HRCT. There was no correlation between circulating fibrocyte counts and radiological severity scores in patients with BPD and IPF [8,13]. As mentioned above these findings could be explained with the migration of circulating fibrocytes to the damaged lungs in CF patients. It was shown that TGF-β1 is increased in BAL and plasma of the CF patients with the infection and lung disease severity [24]. TGF-β1 and other cytokines might promote migration of fibrocytes to the lung parenchyma for myofibroblast transformation and extracellular matrix production. This may explain why circulating fibrocytes are less in patients with apparent lung disease and patients at the exacerbation period. We speculated that our findings suggested that fibrocytes might play a role in the pathophysiology of CF lung disease.

Major limitation of this study is low patient number and lack of BAL fibrocyte levels in stable and exacerbation period of CF patients. Also, tissue biopsies would unequivocally reveal whether circulating fibroblasts in the tissue are elevated or not.

To the best of our knowledge, there is only a single recent study analyzing circulating fibrocyte count in patients with CF, therefore, these findings are expected to shed light on future studies. Further studies are also needed to determine the role of circulating fibrocytes in the pathogenesis of CF.

Abbreviations

ARDS:Acute respiratory distress syndrome

a-SMA:alpha-smooth muscle actin

BAL: bronchoalveolar lavage

BPD:bronchopulmonary dysplasia

CF**:** cystic fibrosis

CFTR: Cystic fibrosis transmembrane regulator (CFTR)

COPD:Chronic obstructive pulmonary disease

DMSO:dimethyl sulfoxide

FACS buffer:flow cytometry staining buffer

FSC-A: Forward Scatter

$FEV_{1:}$ forced expiratory volume in 1 second

FVC: forced vital capacity

HRCT: high-resolution computed tomography

ILD:Interstitial lung disease

IPF: idiopathic pulmonary fibrosis

PBMC: peripheral blood mononuclear cell

PBS: phosphate-buffered saline

PFT : pulmonary function test

SSc: systemic sclerosis

SSC-A:Side Scatter

Declarations

Funding: The study was funded by the Scientific Research Projects Funding of Erciyes University (project no: TTU-2018-8356).

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: The Ethical approval was obtained from the University of Erciyes (2018/207).

Consent to participate: Informed written consent was obtained from the parents of the participating individuals.

Consent for publication: Give my consent for the publication of identifiable details, which can include table(s) and/or figure and/or original article and/or details within the text (“Material”) to be published in the European Journal of Pediatrics.

Availability of data and material: Data available on request from the authors.

Code availability: N/A

Authors' Contributions: MK planned and designed the study. PAY, MH contributed with input to the design and participated in the interpretation of the data. PAY, MK reviewed the literature, organized the writing and wrote the initial manuscript. Radiological images of the patients were evaluated by FK.Serum fibrocyte levels of the patients were investigated by AE, PAY and SE. All authors critically reviewed the manuscript and approved the final version for submission.

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Tables

Table 1: HRCT scoring system for cystic fibrosisa

Category	score			
	0	1	2	3
Severity of bronchiectasis	absent	mild (luminal diameters lightly)	moderate (lumen 2–3 times the diameter of vessel)	Severe (lümen >3 times diameter of vessel)
Peribronchial thickening	absent	mild (wall thickness equal to diameter of adjacent vessel)	moderate (wall thickness greater than and up to twice the diameter of adjacent vessel)	severe (wall thickness>2 times the diameter of adjacent vessel)
Extent of bronchiectasis (no.of BP segments)	absent	1–5	6–9	>9
Extent of mucus plugging	absent	1–5	6–9	>9
Sacculations or abscesses	absent	1–5	6–9	>9
Generations of bronchial divisions involved	absent	upto 4th generation	upto 5th generation	Up to 6th generation and distal
No. of bullae	absent	unilateral (not >4)	bilateral (not >4)	>4
Airtrapping	absent	1–5	>5	
Collapse: consolidation	absent	subsegmental	segmental:lobar	

[a]Calculated points from this table will be subtracted from 25 to determine the patient's HRCT score in order to use the latter with the Shwachman–Kulcyc kiclinical scoring system

Table 2: Demographic information of CF, CF exacerbation and control groups and fibrocyte levels

		CF n (39)	Control n (20)	p
Sex	**Male**	18 (%46)	9 (%45)	0.904
	Female	21 (%54)	11 (%55)	
Age (year) median		9 (3-18)	10 (4-17)	0.124
Fibrocyte number median (min-max)		136862 (7491-7796491)	4141 (333-321547)	*<0.001*
		CF exacerbated patients (n=8)		
		Stable period 8	**During exacerbation 8**	
Fibrocyte number median (min max)		106605 (8900-337176)	4783 (1160-24206)	<0.001

Figures

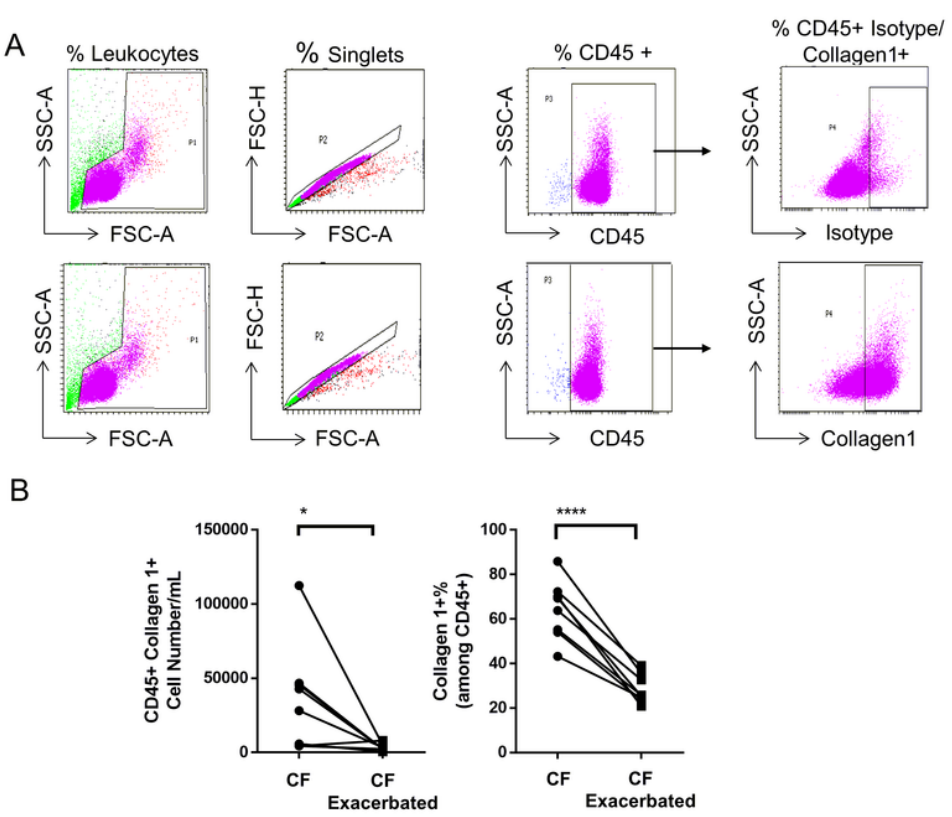

Fig 1

Figure 1

Gating Strategy for Fibrocytes and Fibrocyte levels in CF Patients before and after exacerbation. A) Flow cytometry gating strategy. B) Before and after exacerbation fibrocyte percentage and absolute numbers in CF patientsexamined longitudinally. (n=8), ***indicates p val<0.0001. CF: Cystic Fibrosis

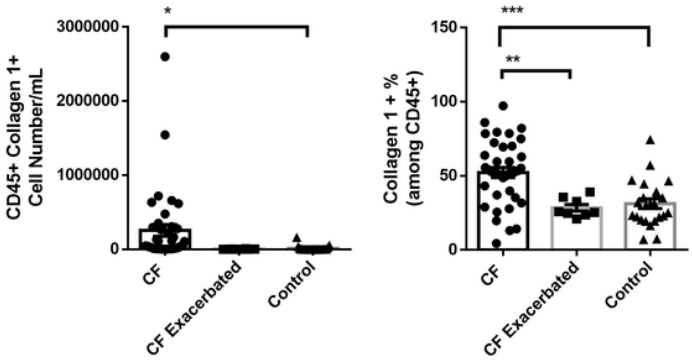

Figure 2

Fibrocyte absolute number and percentages (among CD45+ cells) in CF patients' and healthy control'speripheral blood. CF (n=39), CF exacerbation (n=8), control (n=20), *** indicates p val<0.001. CF: cystic fibrosis