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Overexpression of transforming growth factor (TGF)-β1 and TGF-β3 genes in lung of toxic-inhaled patients

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ABSTRACT

Iraq frequently used toxic inhalants during the war with Iran, exposing over 100,000 people to chemical reagents. Bronchiolitis obliterans (BO) is a major pulmonary disease caused by exposure to harmful gases. Recently defect in clearance of apoptotic cells (efferocytosis) has been suggested as a mechanism that leads to several lung diseases. Transforming growth factor (TGF)-β, a cytokine produced by efferocytotic macrophages, suppresses the inflammation and enhances the regeneration of tissue. In this study, the authors compared the expression of these 3 isoforms of TGF-β at mRNA level in lung biopsies of Iranian victims of chemical gases with lung biopsies of control healthy volunteers. Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) technique was used to examine the expression level of TGF-β isoforms using glutaldehyde 3-phosphate dehydrogenase (GAPDH) gene as an internal control. The results indicated that that levels of TGF- β1 and TGF-β3 mRNAs were significantly higher in chemical gas–injured patients than noninjured group ($P < .05$). Therefore, the authors speculate that TGF-β1 and TGFβ3, but not TGF-β2, secretion is a result of efficient efferocytosis in chemically injured patients, playing a protective role by improving airway remodeling and lung homeostasis in this group. These properties of TGF-β are consistent with long-time survival of chemical-injured people suffering from BO.

KEYWORDS bronchiolitis obliterans (BO), efferocytosis, toxic inhalants, transforming growth factor β (TGF-β)

There are approximately 30,000 survivors that were exposed to toxic inhalants during the Iran-Iraq war (1981–1989). These survivors suffer from late effect of this warfare, including ophthalmic, cutaneous, and respiratory sequels [1]. Among these disorders, the latter is the most lethal illness [2]. Recent surveys have indicated that bronchiolitis obliterans (BO) is the main late respiratory complaint of these patients [3–5], but the BO observed in these patients is somehow different from BO resulted from other causes. For example, unlike post–lung transplant BO, there is no progressive pattern and the severity of fibrosis is completely different and rare [6].

Transforming growth factor beta (TGF-β) is a multifunctional cytokine that controls different sets of processes, including tissue remodeling and repair, cell apoptosis and survival, extracellular matrix production, and inflammation [7]. Three structurally similar isoforms of TGF-β (TGF-β1, -β2, and -β3), encoded by 3 distinct genes, have been identified in mammalian species [8]. These 3 isoforms signal through the same cell surface receptors and have similar cellular targets, although each isoform is expressed in a distinct pattern under control of a unique promoter [9]. TGF-β1 is the prevalent isoform and is found almost ubiquitously, whereas the other isoforms are expressed in a more limited spectrum of cells and tissues. Although the 3 isoforms have similar in vitro properties, their in vivo effects are distinct. Knockout experiments in mice have suggested

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that each TGF-β isoform plays an independent role in embryonic development, underlining their nonredundant functions [10, 11].

TGF-β is produced by lung epithelium, fibroblasts, and smooth muscle cells, and contributes to airway remodeling in chronic lung diseases [12, 13]. It is also produced or released by infiltrating cells such as lymphocytes, monocytes/macrophages, eosinophils, and platelets during tissue fibrosis [14–16]. Efferocytosis, the engulfment of apoptotic cells by phagocytes followed by cell replacement to maintain homeostasis, seems to be necessary for normal function of lung [17, 18], and several lung diseases, including asthma [18], chronic obstructive pulmonary disease (COPD) [19–21], emphysema [19], cystic fibrosis (CF), and non-CF bronchiectasis [21] result from impaired efferocytosis. It has been suggested that by efficient efferocytosis, TGF-β is secreted from efferocytotic macrophages, and acts as an anti-inflammatory and progrowth mediator; however. in disease states such as CF and COPD in which efferocytosis is impaired, the level of TGF-β is decreased [17, 18].

In view of TGF-β properties, we assumed that TGF-β might be responsible for airway remodeling, homeostasis, and slow progression of respiratory disease in chemical-injured patients. Using enzyme-linked immunosorbent assay (ELISA) technique, our team previously showed that the amount of TGF-β1 protein was higher in bronchoalveolar lavage (BAL) aspirates of Iranian war veterans exposed to chemical gases [2], in comparison with the control group; however, the expression of the other 2 isoforms, TGF-β2 and TGF-β3, has not been examined yet. In the present study, in order to clarify the significance of each TGF-β isoform in lung disease of people poisoned by toxic inhalant, we examined the mRNA expression for TGF-β1, TGF-β2, and TGF-β3 genes in lung biopsies of chemical-injured patients by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR), and compared it with noninjured patients.

MATERIALS AND METHODS

Subjects

Fourteen healthy volunteers and 20 patients suffering from late effects of exposure to chemical gases were included in the study. All the healthy subjects were free from respiratory diseases, with normal chest x-ray films, high-resolution computed tomography (HRCT) scan, pulmonary function tests (PFTs), and bronchoscopy. In addition, these patients displayed no respiratory symptoms for at least 3 months before this study. The range of healthy people age (1 female and 13 male) was 43 to 64 years. The case group were patients who had been exposed to chemical gases 15 to 16 years ago during the Iran-Iraq war, and they had clinical signs such as blisters or ocular injuries according to their patient charts. Furthermore, they had shown symptoms of BO confirmed by high-resolution computed tomography (HRCT) scan and biopsy samples taken by bronchoscopy in previous studies [3–5]. All patients were male with the age range of 38 to 56 years. Patients with other chronic lung diseases, autoimmune diseases, chronic infectious diseases, cancers, or acquired autoimmunodeficiency syndrome, as well as smokers, addicts, and patients treated with corticosteroids were excluded from the study.

Fiber optic bronchoscopy

All subjects of the trial were appropriately informed of their situation and the reasons for the recommendations for bronchoscopy were explained, and written consent was obtained from all subjects. The upper respiratory tract was anesthetized with 2% lidocaine. Atropine (0.75 mg intramuscularly) was administered before the procedure. Supplemental oxygen was given throughout the procedure, and the oxygen saturation was monitored by continuous pulse oxymeter. Via a flexible fiber optic bronchoscope (Olympus BF1T, Tokyo, Japan), fiber optic bronchoscopy was performed to obtain right upper lobe lung biopsy specimens, using small pinchers attached to a long cable threaded through the bronchoscope by a specialist. The biopsies were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RT-PCR

Total RNA was isolated from frozen specimens using the high pure RNA tissue kit (Roche, Germany), according to the manufacturer's instructions. Briefly, lung biopsies were homogenized, lysis buffer was added, and centrifuged; total RNA was precipitated by absolute ethanol, incubated with DNase, and centrifuged; finally total RNA was washed by wash buffer, and was eluted by distilled water. The purified RNA was used for first-strand cDNA synthesis, using first-strand cDNA synthesis kit (Cinnagene Iran) by oligo $(dT)_{18}$ primer (MWG, Germany) in a 20-μL reaction according to the manufacturer's instructions. The amplification of the genes of interest was performed in 25-μL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μL of first-strand cDNA, 0.5 U of recombinant Taq DNA polymerase (Cinagene), 200 μM of each

TABLE 1 The Sequences and Other Features of the Primers Employed in This Study

Gene and GeneBank ID	Primer	Primer sequence (5′ to 3′)	Annealing T_m (°C)	PCR product length
TGF-β1 (NM-000660)	Forward	ACCCACAACGAAATCTATGACAAG	60	624
	Reverse	GAGGCAGAAGTTGGCATGGTAG	60	
TGF-β2 (NM-003238)	Forward	AGAAGACTATCCTGAGCCCGAG	59	448
	Reverse	TACATCGAAGGAGAGCCATTCGCC	59	
TGF-β3 (NM-003239)	Forward	CATAAATTCGACATGATCCAGGGG	59	645
	Reverse	GCCATGGTCATCCTCATTGTCCAC	59	
GAPDH (NM-002046)	Forward	CCAGCCGAGCCACATCGCTC	56	359
	Reverse	ATGAGCCCCAGCCTTCTCCAT	56	

deoxynucleoside triphosphate, and 4 μM of each primer (Table 1). PCR was performed under similar conditions for selected genes and the internal control. The initial denaturation was performed at 94°C for 1 minute and amplification was performed by 30 and 35 cycles of denaturation at 94°C for 40 seconds, annealing at 56°C to 60°C for 30 seconds, and extension at 72°C for 60 seconds followed by a 5-minute final extension. PCR products were subjected to agarose gel electrophoresis. The expression level of gene was quantified according to the band intensity on agarose gel stained with ethidium bromide was measured using UVItec software. The identity of PCR products were confirmed by restriction enzyme digestion and also by sequencing (data not shown).

Statistics

Data were shown as mean ± standard deviation (SD). SPSS 15.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. All experiments were repeated twice and the results were analyzed by performing t tests. $P < .05$ was considered as statistically significant.

RESULTS

In total, 20 lung specimens from SM-injured patients and 14 samples from healthy control people were collected. Patient's demographic characteristics are demonstrated in Table 2. Specific primers were designed for amplification of fragments of different TGF-β genes isoforms. The expected band sizes for GAPDH, TGF-β1, -β2, and -β3 were 359, 624, 448, and 465 bp, respectively. The 624-bp PCR product corresponding to amplified TGF-β1 fragment was visualized in 11 out of 14 healthy control samples (78.5%) and in 19 out of 20 (95%) patient samples (Figure 1). The expression level of TGF-β1 in patients poisoned with toxic inhalants appeared to be higher in comparison with controls (Figure 2). Given that the cDNA of TGF-β2 was detectable only as a weak signal in a small number of control and patient samples (data not shown), we ignored the expression analysis of this isoform in our study.

We detected the transcript of TGF-β3 cDNA in 9 out of 14 (~64.3%) control samples and in 14 out of 20 (70%) patient specimens. The results showed that as with TGF-β1, the expression level of TGF-β3 was higher in patients exposed to toxic inhalants than healthy people (Figure 2).

DISCUSSION

According to the last reports, bronchiolitis obliterans (BO) is the main pulmonary disease among the survivors of Iranian veterans and civilians poisoned with toxic inhalants during the Iraq-Iran war [5]. Little is known about the molecular mechanisms leading to structural alterations and pathological symptoms observed in the lungs of these people. Using ELISA technique, we previously detected higher levels of TGF-β1 protein in BAL fluid of a group

TABLE 2 Demographic Characteristics of the Population Studied

	Control ($n = 14$)	Inhalation injury ($n = 20$)
Age range (year)	43 to 64	38 to 56
Sex		
Male (%)	13 (93%)	20 (100%)
Female (%)	1 (7%)	None
Smoking history	None	None
Weight (kg)	70.3 ± 12.5 (45–93)	73.5 ± 12.1 (52–98)
Height (cm)	167.9 ± 9.6 (154–181)	170.4 ± 8.7 (142–184)
BMI (kg/m^2)	25.7 ± 4.9	23.8 ± 5.2
FVC (L)	2.85 ± 0.87	3.34 ± 0.52
FEV_1 (L)	1.9 ± 0.78	2.87 ± 0.7
FEV_1/FVC	67.2 ± 13.1	69.5 ± 15.1

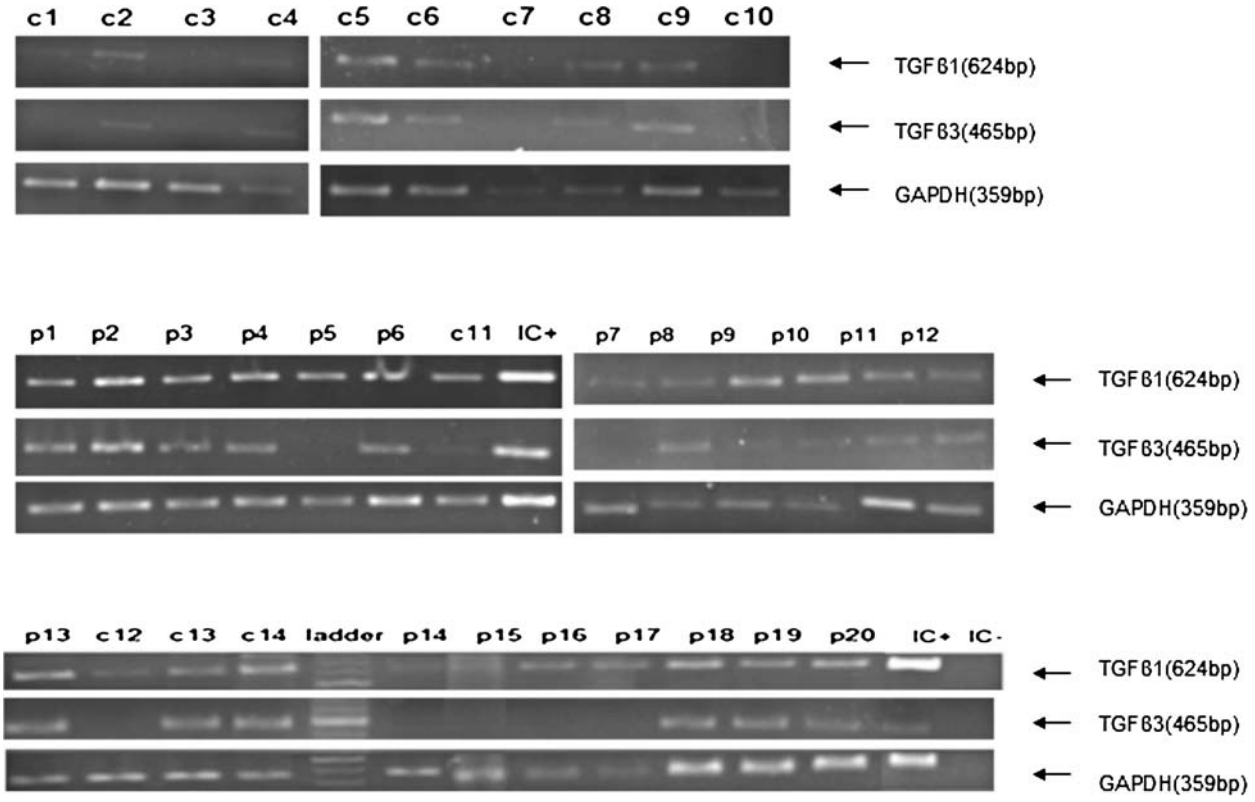

FIGURE 1 Reverse transcriptase–polymerase chain reaction analysis of the expression of TGF-β1 (624 bp), TGF-β3 (465 bp), and *GAPDH* (359 bp) in the lung samples obtained from toxic-inhaled patients (P) and control people (C).

of veterans exposed to chemical gas in comparison with nonexposed subjects [2]. In this study, expression of different types of TGF-β transcripts is examined between Iranian chemically injured patients and healthy volunteers. Our result is consistent with studies done by other groups, in detection of TGF-β1 and TGF-β3 genes expression in normal adult human lung [15, 22, 23]. The transcripts of theses 2 genes were also detected in specimens of toxic inhalant–injured patients. TGF-β1 gene expression appeared to be increased in patients, which is compatible with our previous data [2]. The TGF-β3 gene expression was higher in the patient group, but we could not detect any significant gene expression for TGF-β2 gene in either the patient or the control group. This could be because of weak expression of this isoform in airway of 2 groups, or low sensitivity of our method.

TGF-β appears to have a role in most respiratory disorders [13], and it has been suggested that TGF-β serves as an early marker of BO [24]. In rodent models, the role of TGF-β and its signaling pathway in development of BO has also been reported [25, 26]. El-Gamel et al. showed overproduction of TGF-β in BO patients, but they did not distinguish the exact up-regulated isoform of this factor [27]. Bergman et al. detected slightly increased levels of TGF-β1 transcripts in bronchoalveolar lavage (BAL) cells of lung transplant recipients affected with BO [28]; however, we have found over 3-fold up-regulation of TGF-β1 gene in our patients. Smad pathway is the most represented signaling mechanism for TGF-β; however, it can also activate alternative signaling pathways, including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 mitogen-activated protein kinase (MAPK), which are members of the MAPKs. These MAPK-related pathways can mediate or enhance Smad-dependent responses, or result in Smad-independent effects [29–32]. The availability of active TGF-β1 ligand could be one of the factors determining which downstream pathway is activated. Although extensive post-transcriptional regulation makes it complicated to predict the concentration of active TGF-β1 protein just by measuring the level of its transcripts, we have previously shown higher levels of TGF-β1 protein in BAL samples of our patients, which is consistent with its transcript levels in this group of people. Therefore, different levels of increase in TGF-β1 expression between our patients and patients

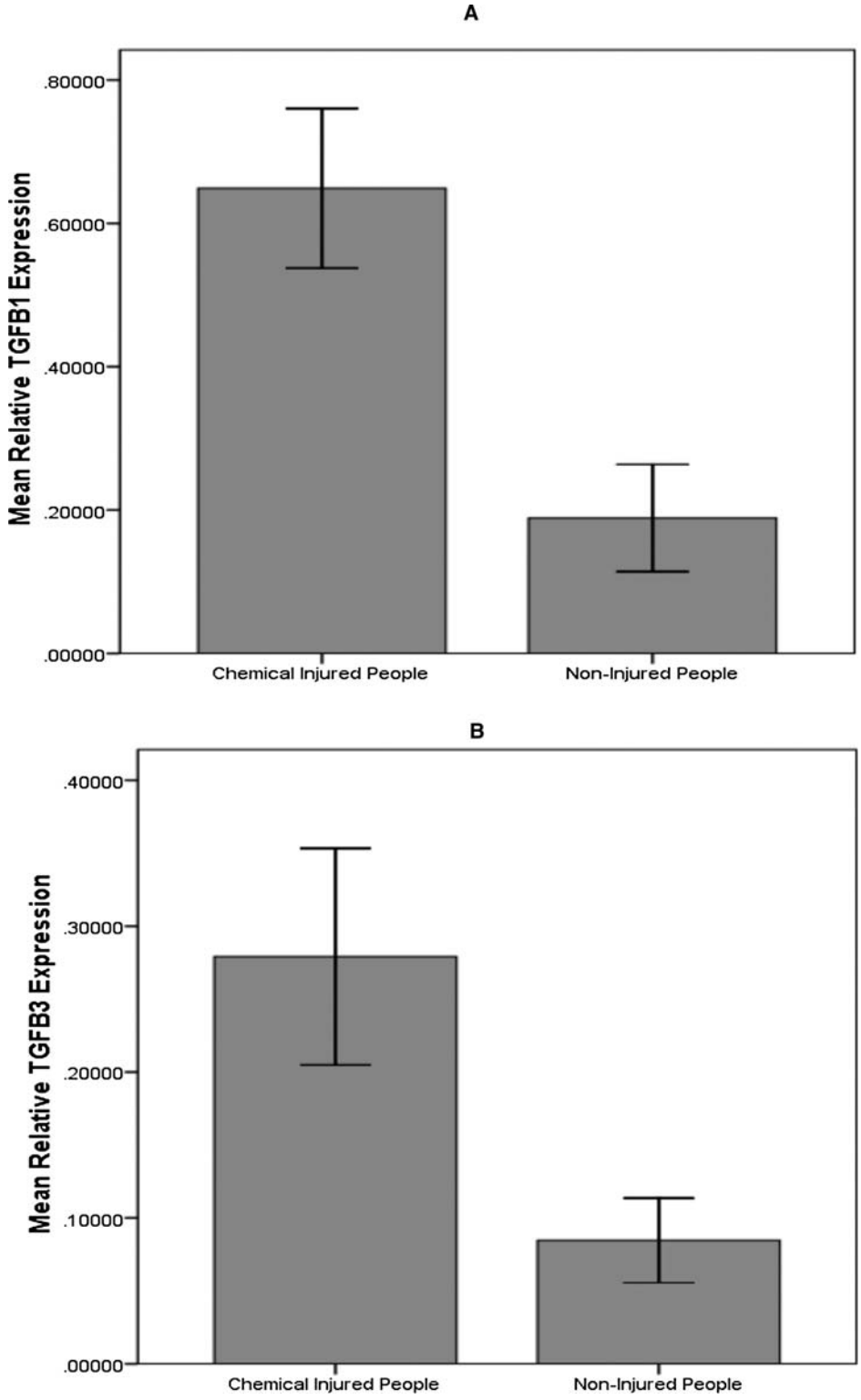

FIGURE 2 Mean Relative intensity of TGF-β1 (**A**) and TGF-β3 expression (**B**) in chemical-injured patients and control people. Relative band intensities for TGF-β1 and TGF-β3 for each sample were quantities by densitometry, normalized to GAPDH expression, and the mean of expression in the different groups is shown as histograms. Statistical analysis revealed that the expression of TGF-β1 and TGF-β3 is significantly higher in chemical-injured samples compared with healthy ones ($P < .001$ for TGF-β1 and $P < .01$ for TGF-β3).

suffering from post–lung transplantation BO raise the possibility that TGF-β1 could have a different function and downstream signaling pathway in each group. However, understanding the exact role of TGF-β has been hampered by the complex and unusual biology of TGF-β activation and by the amazing diversity of its effects, eliciting multiple, and often opposing, cellular responses [33]. For example in some experiments, TGF-β shows anti-inflammatory and immunosuppressive effects [9, 34], whereas it appears to be a proinflammatory factor in others [9, 35]. It has been suggested that TGF-β1 released by the phagocyte acts in an autocrine/paracrine manner to suppress the production of inflammatory cytokines, chemokines, and lipid mediators [36]. In different tissues, including skin and lung, it has been observed that TGF-β1 is involved in wound healing, fibrogenesis, and angiogenesis [37–39]. On the other hand, it seems that by inducing cellular apoptosis and decreasing epithelialization, it could prevent wound repair [40–43]. The complexity of TGF-β effects could be due to several reasons, including different state of activation and differentiation of the target cells, the presence of other stimuli in the local microenvironment, and the presence of different signaling pathways by which TGF-β could exert its antagonistic effects [40–44].

According to several studies, TGF-β plays a pivotal role in extracellular matrix homeostasis. TGF-β can stimulate the expression of tissue inhibitors of matrix metalloproteinase (TIMPs) and extracellular components, and also inhibit the expression of several matrix metalloproteinases (MMPs). One of TGF-β downstream proteins, connective tissue growth factor (CTGF), mediates part of its fibrogenic function. Excessive amount of TGF-β could result in accumulation of extracellular matrix and decreased degradation of it, which could lead to fibrotic diseases of lung, heart, and skin [14, 45–47]. Along with cell proliferation, extracellular matrix deposition seems to have an important role in the pathogenesis of BO (28, 48). However, clinical investigations showed no symptoms of fibrosis in our patients. In order to analyze the presence of fibrosis at the molecular level, and to see if TGF-β is involved in extracellualr accumulation, we evaluated the expression of TIMP1 and CTGF genes in our patient and control groups. We did not see any difference in expression TIMP1 between the 2 groups, and surprisingly we found a slight decrease in CTGF expression in BO patient group (unpublished data). These data suggest that TGF-β is not involved in fibrogenic processes in our patients; however, further expression analysis of other TGF-β downstream genes that are involved in extracelluar matrix homeostasis is suggested to confirm these data. So far, both molecular and clinical studies have indicated the absence of fibrosis in these patients.

Suppression of inflammatory response and improvement of tissue regeneration have recently emerged as protective functions of TGF-β1 [17, 18], which could lead to long-term survival of patients suffering from different lung diseases. Apoptosis is important for the regulation of normal cell turnover in the lung and is a key mechanism in the control of the repair process [49]. Under normal conditions, apoptosis is followed by efferocytosis, which is a rapid and specialized phagocytosis of apoptotic cells by macrophages/monocytes with minimal inflammatory response [50–52]. However, increased rates of apoptosis of lung cells may result in unbalanced homeostasis, leading to an overloading of the local capacity for phagocytosis and defective clearance [49, 53]. Studies have shown that macrophage ingestion of apoptotic cells causes an increased release of TGF-β from these cells [54, 55] that results in suppression of inflammatory and immunogenic response, proliferation of epithelial and endothelial cells, and the maintenance of normal lung structure [52, 56, 57]. These observations are compatible with CF and COPD diseases in which the TGF-β protein level is lower than normal, which could result from ineffective clearance of apoptotic cells, therefore, insufficient levels of TGF-β could lead to sustained inflammation and impaired tissue repair [17, 18].

In spite of the fact that the main pathologic diagnosis in our patients is BO, the declining speed of pulmonary function (PF) is slower than rate seen in BO patients due to other causes [6]. This could be explained by efficient efferocytosis, which is followed by secretion of TGF-β1 and TGF-β3 in the lungs of these patients, leading to suppression of inflammation, less perspective removal of apoptotic cells, and robust maintenance of the balance between cell death and replacement.

CONCLUSION

Considering the data of this study and others we speculate that TGF-β1 and TGF-β3 are anti-inflammatory cytokines, which are secreted by macrophages involved in effercytosis, and are responsible for bland removal of apoptotic cells, and proper lung tissue repair. This is manifested by the slow progression of respiratory disorder, BO, and absence of fibrosis in these people. Herein, we considered only the expression of TGF-β. Further studies consisting of immunohistochemistery (IHC) are suggested to investigate other profibrotic or antiapoptotic genes to improve the hypothesis.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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