Does Defective Apoptosis Play A Role in Cystic Fibrosis Lung Disease?

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Background and Aims. Although apoptotic dysfunction has recently been suggested in cystic fibrosis (CF), there are few studies reported concerning apoptosis in CF with controversial results. The aim of this study was to investigate apoptosis in CF human lung tissues and compare with non-CF bronchiectatic and normal healthy lung tissues. We also investigated the relation between apoptosis and histopathological features of tissues and microbiological factors influencing apoptosis.

Methods. Lung tissue samples from CF (n = 30), non-CF bronchiectasis (n = 28, BE group) and normal control cases (n = 24, C group) were included in the study. Histological examination of H & E-stained archived slides was performed and TUNEL method was used to detect DNA fragmentation.

Results. Apoptotic alveolar epithelial cells were significantly increased in the CF group compared to BE and C groups (p = 0.046). Bronchopneumonia (BP) was present in 15 CF cases (50%), whereas none of the cases in C group had BP (p = 0.0001). Apoptosis was significantly increased in cases with BP (n = 17) compared to cases without BP (n = 65) (p = 0.04).

Conclusions. Apoptotic epithelial cells and BP were significantly increased in the CF group and excess level of apoptosis may be the result of enhanced occurrence of BP. Apoptotic cells were alveolar epithelial cells in the great majority of the patients and were not detected in other locations where CFTR expression is much more prominent than alveolar cells. We may postulate that increased apoptotic findings in the alveolar epithelium were related with the presence of chronic infections rather than CFTR dysfunction. © 2009 IMSS. Published by Elsevier Inc.

Key Words: Apoptosis, Bronchiectasis, Bronchopneumonia, Cystic fibrosis, Lung.

Introduction

Cystic fibrosis (CF) is a single-gene disease and is the most common lethal inherited disease in Caucasians. CF is caused by mutations in the CF transmembrane regulator (CFTR) gene resulting in absent or deficient expression and function of CFTR protein (1,2). CFTR protein is expressed in epithelial cells of the pancreas, intestine, sweat glands, and upper and lower respiratory tracts (3). Although it has been demonstrated that CFTR is a Cl⁻ channel, it has been postulated that CFTR may affect other cellular functions and participate in the apoptotic process by influencing the intracellular pH (1). Apoptosis, programmed cell death, is a physiological process essential for the maintenance of homeostasis of epithelial organization and function for clearance of inflammatory cells (2). Although apoptotic dysfunction in CF has recently been suggested, there are few studies reported concerning apoptosis in CF with controversial results (3–5). A high DNA fragmentation and high level of Fas ligand expression have been reported in both human CF enterocytes and bronchial epithelial cells (1,2). On the other hand, a resistance in the initiation of apoptosis induced by etoposide was reported in a mutant ΔF508 epithelial mouse mammary cell line (6).

The aim of this study was to investigate apoptosis in CF human lung tissues and compare with non-CF bronchiectatic and normal healthy lung tissues. We also planned to investigate the relation between apoptosis and histopathological findings and microbiological factors influencing apoptosis.
Patients and Methods

Between 1979 and 2005, lung tissue samples from CF (25 autopsy cases and 5 lobectomy materials; CF group) and non-CF bronchiectasis (n = 28, BE group) cases who were being followed-up in the Department of Pediatric Chest Diseases and/or had lung tissue samples examined in the Department of Pediatric Pathology at the Faculty of Medicine of Hacettepe University were included in the study. Normal control lung tissue (n = 24, C group) was provided from autopsy material of non-CF patients.

All tissues (autopsy and lobectomy samples) were fixed in buffered formaldehyde at room temperature. No pre-fixation was applied. Fixation time was similar for all samples (1–2 days).

H & E-stained archived slides were examined and major pathological findings were classified as bronchopneumonia (BP), follicular bronchiectasis (FB) or follicular infective bronchiectasis (FIB). The remaining cases showed nonspecific findings (NS).

To detect DNA fragmentation, we used in situ terminal deoxynucleotidyl transferase d uridine triphosphate nick end labelling (TUNEL) method (ApopTag; Qbiogene apoptosis detection kit, Carlsbad, CA). For TUNEL assay, sections provided within the apoptosis kit (ApopTag) were used as positive control sections and positive staining was obtained with these. At least 20 fields were examined for each case under 20× magnification, and bronchi, bronchioles and submucosal glands were investigated for TUNEL-positive apoptotic cells. Apoptotic cells were alveolar epithelial cells in the great majority of the patients. Because the ratio of apoptotic cells to all epithelial cells was <10% in each slide, apoptotic activity was not further graded and reported only as positive or not (7).

Statistical Analysis

The sputum culture positivity, histopathological findings, presence of apoptotic cells and factors which might affect apoptosis were compared in 3 groups using the χ² test with a statistical software package (SPSS, v.11; SPSS Inc; Chicago, IL). In each situation, a p value of <0.05 was considered significant.

Results

Patient Characteristics

CF group consisted of 30 patients with a median age of 3 months (range: 4 days to 15 years). This group included 12 girls and 18 boys. There were 28 cases (male/female = 13/15) in the BE group who had a median age of 9.1 years (range: 18 months to 15 years). The etiologies for bronchiectasis were immune deficiencies (n = 3), primary ciliary dyskinesia (n = 3), Kartagener’s syndrome (n = 2) and unidentified in the remaining 20 cases. C group

Table 1. Demographic, clinical, histopathological and apoptotic features of patients.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CF (n = 30)</th>
<th>Bronchiectasis (n = 28)</th>
<th>Control (n = 24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12 (40%)</td>
<td>15 (54%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>18 (60%)</td>
<td>13 (46%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ages</td>
<td>4 days–15 years</td>
<td>18 months–15 years</td>
<td>1 month–6 years</td>
<td></td>
</tr>
<tr>
<td>Median age</td>
<td>3 months</td>
<td>9.1 years</td>
<td>16 months</td>
<td></td>
</tr>
<tr>
<td>Histopathological findings (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP positive</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>0.0001</td>
</tr>
<tr>
<td>FB positive</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>FIB positive</td>
<td>1</td>
<td>19</td>
<td>0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Apoptotic cells (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>14 (47%)</td>
<td>5 (18%)</td>
<td>6 (25%)</td>
<td>0.046</td>
</tr>
<tr>
<td>Negative</td>
<td>16 (53%)</td>
<td>23 (82%)</td>
<td>18 (75%)</td>
<td></td>
</tr>
<tr>
<td>Culture positive (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔF508 Mutation (%)</td>
<td>30%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CF, cystic fibrosis; BP, bronchopneumonia; FB, follicular bronchiectasis; FIB, follicular infective bronchiectasis.

Figure 1. Apoptotic alveolar epithelial cells shown with TUNEL method. The ratio of TUNEL-positive cells to all epithelial cells was <10% in all cases. Color version of this figure available online at www.arcmedres.com.
Histopathological Findings

BP was present in 15 CF cases (50%) and two BE cases (7%); none of the cases in C group had BP (p = 0.0001). FB was not detected in any of the patients in CF or C group, whereas seven cases (25%) in BE group had FB (p = 0.001). FIB findings were detected in only one patient in CF group (3.3%) compared to 19 cases (67.9%) in BE group; none of the cases in C group had FIB (p = 0.0001). Histopathological characteristics of lung tissues in various groups are given in Table 1.

Apoptotic Findings

Apoptotic cells were detected in 14 cases with CF (47%), five cases with BE (18%) and six cases in C group (25%). Apoptotic cells were significantly increased in the CF group compared to BE and C groups (p = 0.046). No significant difference was detected between the BE and C groups with regard to presence of apoptotic cells (p = 0.7). Apoptosis was detected in a few bronchial epithelial cells of three CF cases and two BE cases, whereas none had apoptotic cells in the glandular epithelium. In all remaining cases, apoptotic cells were found in the alveolar epithelium (Figure 1). Apoptotic findings are displayed in Table 1.

Factors Affecting Apoptosis

In 25 patients, apoptosis was detected in the lung tissues (14 CF, 5 BE, 6 C cases), whereas BP findings were present in 17 cases (15 CF, 2 BE cases) (Tables 1 and 2). Apoptotic findings were significantly increased in cases with BP findings (n = 17) compared to cases without (n = 65) (p = 0.04). This was regarded as evidence of a positive relation between the presence of apoptotic cells and BP in the lungs (Table 2 and Figure 2). No significant relation was demonstrated between the bacterial growth rate, presence of FIB or FB and presence of apoptosis.

Discussion

This study demonstrated that apoptotic epithelial cells and BP findings were significantly increased in the CF group and an excess level of apoptosis may be the result of enhanced occurrence of BP findings. It is known that extracellular microorganisms induce apoptosis on airway epithelial cells during pneumonia (8). In our study, S. aureus and P. aeruginosa were the most common microorganisms identified in CF patients. Lung of CF patients are often exposed to chronic and persistent pulmonary infections with these two pathogens. Kahl et al. (9) infected a respiratory epithelial cell line with S. aureus RN6390, which was derived from a CF patient. These authors showed that it replicated and induced apoptosis in pulmonary epithelial cell line. On the other hand, the ability of P. aeruginosa to induce apoptosis in airway epithelial cells was found to be dependent upon properties of host epithelial cell structure and CFTR genotype of the infected cells. The 9HTEo cells that lacked tight junction integrity were readily susceptible to apoptosis after exposure to P. aeruginosa. However, cultured cells expressing ΔF508 CFTR showed a delayed apoptotic response to this microorganism compared with cells expressing wild-type CFTR (10,11). With prolonged exposure of epithelial cells to P. aeruginosa and in destroyed epithelial structure in CF lung disease, P. aeruginosa-induced apoptosis may be expected in our CF patients (11).

Another important result from our study is that apoptotic cells were detected in alveolar epithelium in all but five cases and none of those had apoptotic cells in the bronchial, bronchiolar or submucosal glandular epithelium. If increased apoptosis was associated with CFTR dysfunction in the lungs, we would have expected apoptotic cells in bronchial, bronchiolar and submucosal gland epithelial cells where CFTR expression is much more prominent than alveolar cells (12). Considering this, we might postulate that the increased.
apoptotic findings in the alveolar epithelium were related with the presence of chronic infections rather than CFTR dysfunction, which is the basic defect in CF.

The effects of CFTR dysfunction on apoptosis have been studied both in vitro and in clinical specimens. Maiuri et al. (1) showed that bronchial \( n = 2 \) and duodenal \( n = 14 \) biopsy specimens from CF patients had more apoptotic cells detected by TUNEL method as compared with controls, and apoptosis was particularly prominent in areas with high CFTR expression, such as the submucosal cells. This study showed that inappropriately high DNA fragmentation may be a feature of various CF epithelia. However, in that study the number of cases is small to draw such a conclusion. On the contrary, a mouse mammary epithelial cell line (C127) transfected with mutant CFTR was found to be resistant to apoptosis, as compared with cells expressing the wild-type gene (6). The decrease in the rates of apoptosis was suggested to be due to consequences of Cl\(^{-}\) channel dysfunction and lack of acidification in the cytoplasm required for caspase activation. Functional CFTR is required for cytoplasmic acidification, which has been observed as an early event in the apoptotic cascade due to the activation of an acid endonuclease required for DNA cleavage (11). Maiuri et al. tried to explain the discrepancy between these studies by stating that the cells may undergo DNA nicking, as seen by the TUNEL assay. However, final degradation by endonucleases does not occur, or other apoptosis related processes fail to occur. This would indicate a situation in which the apoptotic process is initiated but then aborted (4,5,11).

Increased susceptibility to apoptosis in epithelial cells and failed apoptosis in neutrophils would contribute to the self-perpetuating inflammatory cycle in CF. Independent of the susceptibility to apoptosis of CF cells, it has been shown that clearance of apoptotic cells is defective and that accumulation of such cells may contribute to ongoing inflammation in CF patients (5).

The main limitation of our study was based on the TUNEL method that is sensitive, reproducible, and the prevalent method to detect apoptosis in tissues. However, it only detects DNA fragmentation and is known to be positive also in some necrotic cells (its lack of specificity) (1,4,5,13). Used methods may influence TUNEL results. Tateyama et al. (14) investigated effects of prefixation and fixation times on apoptosis detection by in situ end-labeling of fragmented DNA. They found that prefixation time affects the precise identification of apoptosis by the TUNEL method. Longer time prefixation intervals cause an incorrect evaluation with many more apoptotic cells than expected (increased false positivity). Prefixation was not applied in our study, so probable false positivity of TUNEL method could not be attributed to this. Furthermore, in that study (14), the length of fixation time in formalin seemed to have no effect on the results obtained by this method.

Because of some pitfalls of the TUNEL methods, larger studies using more sophisticated techniques to detect apoptosis are required to reveal more precise results. Thus, the exact pathological mechanisms that play a role in the apoptotic processes in CF lung can be elucidated.

References