Inflammatory cells and apoptosis in respiratory and limb muscles of patients with COPD

Esther Barreiro,1,2,3 Dolores Ferrer,4,5 Francisco Sanchez,1 Joan Minguella,5,6 Judith Marin-Corral,1,5 Juana Martinez-Llorens,1 Josep Lloreta,2,4 and Joaquin Gea1,2,3
1Pulmonology Department-Muscle Research and Respiratory System Unit, 4Department of Pathology, and 6Surgery Department, IMIM-Hospital del Mar, Parc de Recerca Biomédica de Barcelona (PRBB), Barcelona, Catalonia; 2Department of Health and Experimental Sciences, Universitat Pompeu Fabra, PRBB, Barcelona, Catalonia; 3Centro de Investigación en Red de Enfermedades Respiratorias, Instituto de Salud Carlos III, Bunnyola, Majorca, Balearic Islands; and 5School of Medicine, Universitat Autònoma de Barcelona, Barcelona, Catalonia, Spain

Submitted 27 August 2010; accepted in final form 13 May 2011

IN PATIENTS WITH SEVERE CHRONIC obstructive pulmonary disease (COPD), skeletal muscle dysfunction, which has been associated with increased mortality (38), is of multifactor etiology (1, 2, 4–6, 9, 10, 13, 19, 21, 26). Interestingly, factors such as hypoxia, oxidative stress, nutritional depletion, deconditioning, and apoptosis, which may also lead to muscle atrophy, are proposed contributors (1, 2, 4–6, 9, 10, 21). Additionally, systemic inflammation, partly originated by the spillover of oxidants and inflammatory molecules from the lungs, has been suggested to contribute to muscle wasting and dysfunction in COPD. For instance, serum levels of tumor necrosis factor (TNF)-α, its soluble receptors I and II, interleukin (IL)-6, and IL-8 along with acute-phase reactants were shown to be increased in patients with severe COPD and muscle wasting compared with patients with identical disease severity without weight loss (18, 35, 39, 40).

At the local level, the vastus lateralis of severe COPD patients with normal weight and no signs of muscle atrophy exhibited greater protein content of TNF-α and larger inflammatory cell counts (26), as well as increased levels of apoptosis (1) than control subjects. Interestingly, no significant differences were found in the levels of molecular or cellular inflammation of the vastus lateralis muscle between low- and normal-weight COPD patients (26). Other studies, however, have shown no TNF-α expression in limb muscles of COPD patients or control individuals with normal body composition (17, 21). Gosker et al. (19) also reported that numbers of macrophages and leukocytes and of apoptotic nuclei were low in the vastus lateralis of severe COPD patients and did not differ from levels in the control muscles. In line with this, we have recently shown (8, 10) that protein levels of TNF-α, IL-6, TNF-α receptor II, and vascular endothelial growth factor (VEGF) were not modified or rather decreased in the vastus lateralis of severe COPD patients compared with control individuals. Moreover, inflammatory cell counts were only slightly increased in the vastus lateralis of a relatively small group of severe COPD patients compared with healthy controls (8). Levels of apoptosis, however, were not explored in this study (8). Apoptosis is a relevant cellular process that contributes to fiber atrophy in peripheral muscles of COPD patients. Mechanisms such as muscle oxidative stress and inflammation may trigger apoptosis within those muscles (11, 14). On the other hand, it also remains to be investigated whether inflammatory cell infiltration and apoptosis develop in the respiratory muscles of COPD patients. On the basis of this, we hypothesized that, in respiratory and limb muscles of severe COPD patients with normal body composition and no signs of muscle atrophy, inflammatory cell counts and apoptosis would be similar to those encountered in a group of healthy subjects. It should also be underscored that the present investigation focuses on the study of patients with COPD exhibiting muscle dysfunction and normal body composition, since this specific population...
constitutes an excellent target for the action of future therapeutic interventions.

Accordingly, in this study, levels of inflammatory cells and apoptosis were quantified in both respiratory and limb muscles of COPD patients with muscle dysfunction and preserved body composition. Hence, our objectives were to determine levels of inflammatory cell infiltration (leukocytes and macrophages) as well as muscle structure in the vastus lateralis, external intercostal, and diaphragm of patients with moderate and severe COPD and in control subjects. Furthermore, in a subgroup of severe COPD patients, moderate COPD patients, and control individuals, we also explored levels of apoptotic nuclei in the vastus lateralis and diaphragm using several methodologies, including ultrastructural analysis.

**MATERIALS AND METHODS**

See additional information in the online supplement (Supplemental data for this article may be found on the Journal of Applied Physiology website.).

**Patients and Control Subjects**

Thirty-one Caucasian male patients with stable COPD (8 moderate and 23 severe patients) (29) exhibiting normal body composition and 17 age-matched sedentary controls were consecutively recruited on an outpatient basis from the COPD and Lung Cancer clinics at Hospital del Mar. Muscle specimens were obtained from the vastus lateralis and external intercostal in all COPD patients and control subjects. However, from the same series of participants, muscle sample specimens from the diaphragm could only be obtained in 10 severe and 8 moderate COPD patients and 10 control subjects during thoracotomy for a localized lung neoplasm.

The current investigation was designed in accordance with both the ethical standards on human experimentation in our institution and the World Medical Association guidelines for research on human beings. The Ethics Committee on Human Investigation at IMIM-Hospital del Mar in Barcelona approved all experiments. Informed written consent was obtained from all individuals.

**Clinical, Nutritional, and Functional Assessment**

Anthropometrical and functional status was evaluated in all individuals as formerly described (16, 37). Briefly, anthropometrical evaluation included body mass index (BMI), fat-free mass index (FFMI) using bioelectrical impedance, and analytical parameters. Forced spirometry and determination of static lung volumes, carbon monoxide transfer, and arterial blood gases were performed using standard procedures, and reference values by Roca et al. (31–33) were used. Inspiratory muscle strength was assessed through determination of maximal inspiratory pressure (MIP) at the mouth (Sibelmed-163; Sibel, Barcelona, Spain) during an occluded maneuver from residual volume. Quadriceps strength was evaluated in both patients and controls by isometric maximum voluntary contraction (QMVC) of the dominant lower limb as formerly described (15).

**Biopsies**

Muscle samples from vastus lateralis and external intercostals were obtained using open surgical biopsy procedures (5–7, 9, 10, 13, 30), whereas diaphragm specimens were obtained during thoracotomy (4, 24). Briefly, biopsy sites were anesthetized previously with 2% lidocaine, and 1- to 1.5-cm skin incisions were made in each case. Muscle samples, 20- to 30-mg size on average, were all obtained below the vastus lateralis fascia through the same incision. Biopsies from the external intercostals (15- to 20-mg size) were taken along the anterior axillary line at the sixth intercostal space following procedures published elsewhere (7, 12, 13). During thoracotomy, because of localized lung lesions, diaphragm biopsy specimens (30- to 40-mg size) were obtained from the interior costal diaphragm lateral to the insertion of the phrenic nerve (4, 24, 27, 28).

All muscle samples were either immediately frozen in liquid nitrogen and subsequently stored at −80°C or immersed in an alcohol-formol bath for 2 h to be thereafter embedded in paraffin. Frozen tissues were used for immunoblotting techniques (caspase-3), whereas paraffin-embedded tissues were used for the assessment of inflammatory cell infiltration, myosin heavy chain isofoms, and apoptosis (immunohistochemical analysis). Moreover, a fragment of the muscle specimens was fixated in glutaraldehyde for the ultrastructural assessment of apoptosis in those sample specimens of bigger size (diaphragms and vastus lateralis of 10 severe COPD patients, 8 moderate COPD patients, and 10 control subjects).

**Muscle Biology Analyses**

Inflammatory cell counts and muscle structure were evaluated in 31 vastus lateralis and external intercostal muscles of COPD patients (8 moderate and 23 severe) and in the same muscles of 17 control subjects, as well as in the diaphragms of both 18 COPD patients (8 moderate and 10 severe) and 10 control individuals. Furthermore, muscle apoptosis was evaluated using four different methodologies (see below) in the vastus lateralis and diaphragms of 10 severe COPD patients, 8 moderate COPD patients, and 10 control subjects.

**Muscle inflammatory cells.** Immunohistochemical analyses were conducted in vastus lateralis, external intercostal, and diaphragm muscles of moderate and severe COPD patients and control subjects following similar methodologies published elsewhere (8, 19, 26). Briefly, on 3-μm muscle paraaffin-embedded sections, leukocytes (anti-CD45 antibody, clones 2B11 and PD7/26; Dako Cytomation, Carpinteria, CA) and macrophages (anti-CD68 antibody, clone PG-M1; Dako Cytomation) were identified following our regular immunohistochemical procedures. Results corresponding to inflammatory cell counts were expressed in two different ways as follows: 1) the ratio of either leukocyte or macrophage numbers to total muscle section area in square millimeter and 2) the ratio of either leukocyte or macrophage cell counts to total number of fields in each muscle section. Moreover, the ratios of total inflammatory cell counts (both leukocytes and macrophages) to either total muscle section area in square millimeters or to total number of fields in each muscle section were also calculated. In this study, we chose as a reference the total muscle section area or the total number of fields in that section, and not the total volume, since the size (any of its 3 dimensions) of either leukocytes or macrophages was bigger than the size of the muscle slides (3 micrometers).

**Muscle fiber counts and morphometry.** Morphometric analyses were carried out in the three muscles of both COPD patients and control subjects following methodologies previously published (4, 5, 8, 24). The cross-sectional area, mean least diameter, and proportions of type I and type II fibers were assessed under light microscopy.

**Assessment of apoptotic nuclei using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay.** In paraffin-embedded sections of diaphragm and vastus lateralis specimens, apoptotic nuclei were identified using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay following precisely the manufacturer’s instructions and previously published studies (1, 19). TUNEL-positive nuclei were those clearly located within the muscle fiber boundary in each section. In each muscle cross section, the TUNEL-positive nuclei and the total number of nuclei were counted blind by two independent observers who were previously trained for that purpose. On this basis, in each muscle preparation, apoptotic fibers were expressed as the percentage of the TUNEL-positive nuclei from the total number of counted nuclei following previously published methodologies (19, 41). A minimum amount of 300 nuclei was counted in each muscle preparation. Final results correspond to the mean values of the counts provided by the two
Table 1. Anthropometric characteristics and functional status of all the study groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control Subjects</th>
<th>Patients with Moderate COPD</th>
<th>Patients with Severe COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )</td>
<td>17 (9)</td>
<td>8 (4)</td>
<td>23 (10)</td>
</tr>
<tr>
<td>Age, yr</td>
<td>65 (9)</td>
<td>66 (4)</td>
<td>67 (5)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.1 (2.8)</td>
<td>26.5 (2.5)</td>
<td>26.9 (3.5)</td>
</tr>
<tr>
<td>FFMI, kg/m²</td>
<td>21.1 (1.5)</td>
<td>20.4 (1.4)</td>
<td>21.0 (1.9)</td>
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<td>FEV₁, %pred</td>
<td>91 (12)</td>
<td>62 (5)***</td>
<td>33 (10)**</td>
</tr>
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<td>FVC, %pred</td>
<td>92 (12)</td>
<td>73 (10)***</td>
<td>52 (12)**</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>75 (3)</td>
<td>61 (7)**</td>
<td>45 (9)**</td>
</tr>
<tr>
<td>RV, %</td>
<td>99 (20)</td>
<td>138 (37)**</td>
<td>194 (66)**</td>
</tr>
<tr>
<td>TLC, %pred</td>
<td>95 (10)</td>
<td>99 (17)</td>
<td>113 (26)**</td>
</tr>
<tr>
<td>RV/TLC</td>
<td>40 (6)</td>
<td>54 (6)**</td>
<td>66 (9)**</td>
</tr>
<tr>
<td>FRC, %pred</td>
<td>92 (21)</td>
<td>110 (24)</td>
<td>151 (45)**</td>
</tr>
<tr>
<td>DLco, %pred</td>
<td>95 (17)</td>
<td>82 (11)*</td>
<td>70 (22)**</td>
</tr>
<tr>
<td>KCO, %pred</td>
<td>90 (15)</td>
<td>86 (8)</td>
<td>78 (20)*</td>
</tr>
<tr>
<td>PaO₂, kPa</td>
<td>12.2 (0.8)</td>
<td>11.0 (0.9)*</td>
<td>8.6 (0.7)***</td>
</tr>
<tr>
<td>PaCO₂, kPa</td>
<td>5.0 (0.5)</td>
<td>5.4 (0.6)</td>
<td>5.9 (0.8)**</td>
</tr>
<tr>
<td>MIP, %pred</td>
<td>91 (25)</td>
<td>71 (17)</td>
<td>57 (20)**</td>
</tr>
<tr>
<td>Pdimax, cmH₂O</td>
<td>NA</td>
<td>–75 (27)</td>
<td>–70 (11)</td>
</tr>
<tr>
<td>Rdimax, cmH₂O</td>
<td>NA</td>
<td>102 (20)</td>
<td>85 (17)†</td>
</tr>
<tr>
<td>QMVC, kg</td>
<td>38.5 (3.7)</td>
<td>33.8 (1.0)**</td>
<td>28.6 (1.6)**</td>
</tr>
</tbody>
</table>

Data are presented as means (SD); \( n \), no. of subjects. COPD, chronic obstructive pulmonary disease; BMI, body mass index; FFMI, fat-free mass index; FEV₁, forced expiratory volume in 1 s; pred, predicted; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; FRC, functional residual capacity; DLco, carbon monoxide transfer; KCO, Krogh transfer factor; PaO₂, arterial oxygen partial pressure; PaCO₂, arterial carbon dioxide partial pressure; MIP, maximal inspiratory pressure; Pdimax, maximal esophageal pressure; Pdmax, maximal transdiaphragmatic pressure; QMVC, quadriceps isometric maximum voluntary contraction. Statistical significance is expressed as follows: \( P < 0.05, * \); \( P < 0.01, ** \); \( P < 0.001, *** \); \( P < 0.05 \), †; \( P < 0.01 \), ††; \( P < 0.001 \), †††.

RESULTS

Characteristics of the Study Subjects

Table 1 indicates the main characteristics of all of the study subjects, whereas Table 2 reveals the main clinical features of patients and control subjects who specifically underwent thoracotomy. No significant differences in age or nutritional status, as assessed by body and fat-free mass indexes (BMI and FFMI, respectively), were observed between control subjects and either severe or moderate COPD patients. However, lung function parameters and arterial blood gases were modified significantly in both moderate and severe COPD patients compared with controls. Global respiratory muscle strength, maximal transdiaphragmatic pressure, and lung function were impaired in both groups of COPD patients compared with the control subjects. The degree of severity in the lung function parameters was more pronounced in the severe COPD group compared with the moderate COPD group.

Table 2. Anthropometric characteristics and functional status of all the subjects undergoing thoracotomy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control Subjects</th>
<th>Patients with Moderate COPD</th>
<th>Patients with Severe COPD</th>
</tr>
</thead>
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<tr>
<td>( n )</td>
<td>10 (10)</td>
<td>8 (4)</td>
<td>10 (10)</td>
</tr>
<tr>
<td>Age, yr</td>
<td>63 (10)</td>
<td>66 (4)</td>
<td>68 (4)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.0 (3.6)</td>
<td>24.9 (2.5)</td>
<td>27.3 (3.2)</td>
</tr>
<tr>
<td>FFMI, kg/m²</td>
<td>21.0 (1.9)</td>
<td>20.4 (1.4)</td>
<td>21.7 (1.7)</td>
</tr>
<tr>
<td>FEV₁, %pred</td>
<td>90 (9)</td>
<td>62 (5)***</td>
<td>41 (7)**</td>
</tr>
<tr>
<td>FVC, %pred</td>
<td>87 (9)</td>
<td>73 (10)**</td>
<td>56 (6)**</td>
</tr>
<tr>
<td>RV, %</td>
<td>91 (19)</td>
<td>138 (37)**</td>
<td>173 (62)**</td>
</tr>
<tr>
<td>TLC, %pred</td>
<td>90 (8)</td>
<td>99 (17)</td>
<td>104 (20)†</td>
</tr>
<tr>
<td>RV/TLC</td>
<td>39 (8)</td>
<td>54 (6)**</td>
<td>64 (11)**††</td>
</tr>
<tr>
<td>FRC, %pred</td>
<td>91 (21)</td>
<td>110 (24)</td>
<td>132 (43)**††</td>
</tr>
<tr>
<td>DLco, %pred</td>
<td>88 (13)</td>
<td>82 (11)*</td>
<td>80 (19)</td>
</tr>
<tr>
<td>KCO, %pred</td>
<td>91 (16)</td>
<td>86 (8)</td>
<td>94 (17)</td>
</tr>
<tr>
<td>PaO₂, kPa</td>
<td>12.2 (0.8)</td>
<td>11.0 (0.9)*</td>
<td>8.8 (0.8)**</td>
</tr>
<tr>
<td>PaCO₂, kPa</td>
<td>5.0 (0.5)</td>
<td>5.4 (0.6)</td>
<td>5.7 (0.6)†</td>
</tr>
<tr>
<td>MIP, %pred</td>
<td>88 (21)</td>
<td>75 (17)</td>
<td>60 (16)*</td>
</tr>
<tr>
<td>Pdimax, cmH₂O</td>
<td>NA</td>
<td>–75 (27)</td>
<td>–70 (11)</td>
</tr>
<tr>
<td>Rdimax, cmH₂O</td>
<td>NA</td>
<td>102 (20)</td>
<td>85 (17)†</td>
</tr>
<tr>
<td>QMVC, kg</td>
<td>38.5 (1.6)</td>
<td>33.8 (1.0)**</td>
<td>29.9 (1.0)**</td>
</tr>
</tbody>
</table>

Data are presented as means (SD); \( n \), no. of subjects. Statistical significance is expressed as follows: \( P < 0.05, * \); \( P < 0.01, ** \); \( P < 0.001, *** \); \( P < 0.05 \), †; \( P < 0.01 \), ††; \( P < 0.001 \), †††.
pressure, and quadriceps strength were reduced significantly in severe COPD patients compared with control subjects.

Inflammatory Cells and Muscle Structure

As shown in Supplemental Table E1 and Fig. 1A, total inflammatory cell counts in the three muscles were very low in all three groups of subjects. In diaphragm and intercostal muscles, inflammatory cell numbers did not differ significantly between COPD patients and control subjects. In the vastus lateralis, inflammatory cell counts, although very low in all groups, were significantly greater in the severe COPD patients than in the controls (Supplemental Table E1 and Fig. 1B). Among the COPD patients, no significant correlations were detected between muscle cellular inflammation levels and either respiratory or muscle strength (Supplemental Fig. E1, A–C).

In the diaphragms of severe COPD patients, the proportion of type I fibers was significantly greater than in muscles of moderate COPD patients or control subjects (Supplemental Table E2). The size of either type I or type II diaphragm fibers did not differ significantly among the three study groups (Supplemental Table E2). The external intercostal muscle did not exhibit any significant variation in either proportions or sizes of the fiber types among the study groups (Supplemental Table E2). Interestingly, in the vastus lateralis, the proportion of type I fibers was significantly reduced in patients with moderate and severe COPD compared with the controls. However, no significant variations were observed in the size of

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Fig. 1. A: representative examples of the localization of a leukocyte [anti-CD45 antibody, clones 2B11 and PD7/26 (Dako Cytomation) ×400, light microscopy] and a macrophage [anti-CD68 antibody, clone PG-M1 (Dako Cytomation), ×400, light microscopy] in the vastus lateralis of a severe chronic obstructive pulmonary disease (COPD) patient. Note that the concentration of inflammatory cells is extremely low in the muscle cross sections. Arrows point toward a leukocyte (left) and a macrophage (right). B: mean values and SD of total inflammatory cell counts are depicted. Although muscle inflammatory cell infiltration was very low in the quadriceps of the study groups, severe COPD patients (n = 23) exhibited a significant increase (*P < 0.05) in the no. of inflammatory cell counts, as measured by inflammatory cells/muscle section area in mm² (left) and inflammatory cells/muscle section area in fields (right) compared with moderate COPD patients (n = 8) and healthy controls (n = 17). ns, Not significant.
either type I or type II fibers in the quadriceps of any of the study subjects (Supplemental Table E2).

Assessment of Muscle Fiber Apoptosis

Nuclei positively stained for the TUNEL assay are shown in Fig. 2, A-D. In severe COPD patients, the diaphragm and vastus lateralis exhibited greater percentages of TUNEL-stained nuclei than in control subjects (Fig. 2E). Moreover, levels of TUNEL-stained nuclei were significantly higher in the diaphragms of both moderate and severe COPD patients and in vastus lateralis of severe patients compared with control subjects (Fig. 2E). Among all the COPD patients, a significant
negative correlation was found between quadriceps TUNEL-stained nuclei levels and the muscle function (Fig. 2F). Ultrastructural signs of muscle apoptosis are shown in Fig. 3, A–C. Interestingly, in the diaphragm or vastus lateralis of severe and moderate COPD patients, numbers of apoptotic nuclei, bearing either early or advanced ultrastructural signs of apoptosis, were not different from those encountered in the control muscles (Fig. 3D and Supplemental Table E3). Likewise, the percentage of muscle fibers positively stained for cleaved caspase-3 did not differ between patients and control subjects in either respiratory or limb muscles (Fig. 4, A and B, and Supplemental Table E3). Finally, protein levels of the percentage of the ratio of cleaved caspase-3 to procaspase-3 did not differ significantly between any group of COPD patients and controls in either respiratory or limb muscles.

DISCUSSION

The main findings in this study are that, in respiratory and limb muscles of patients with COPD exhibiting muscle dysfunction and preserved body composition compared with muscles in healthy control individuals: 1) inflammatory cell numbers in the diaphragm and intercostal muscles did not differ significantly between COPD patients and control subjects, 2) in the vastus lateralis, inflammatory cell counts, although very low, were significantly greater in severe COPD patients than in the controls, 3) levels of TUNEL-positive nuclei were increased significantly in the diaphragms of moderate and severe COPD patients and in vastus lateralis of severe patients, 4) a significant inverse correlation was observed between TUNEL-positive nuclei in the vastus lateralis and quadriceps strength, and 5) ultrastructural signs of apoptosis did not differ between severe COPD patients and control subjects in either respiratory or limb muscles.

Cellular Inflammation and Muscle Structure and Function

Systemic inflammation has been repeatedly associated with muscle loss and dysfunction in patients with COPD. In this regard, blood levels of TNF-α and other inflammatory cytokines along with acute-phase reactants were increased in patients with severe COPD and muscle wasting compared with patients with identical disease severity without weight loss (18, 35, 39, 40). However, it remains contentious whether cellular local inflammation occurs in the muscles of COPD patients and to what extent it could be a contributor to the muscle dysfunction of patients with normal muscle mass and body weight. For
instance, a previous study (26) demonstrated a large increase in TNF-α levels and other inflammatory parameters, as well as in leukocyte counts in the quadriceps muscles of patients with severe COPD with normal BMI. Conversely, other studies have shown no immunohistochemical (21) or mRNA expression of TNF-α (17), together with very low levels of neutrophils in the quadriceps of patients with severe COPD and normal weight, with and without hypoxemia, either at rest or after local exercise (21). More recently, we have shown (10) that, in the vastus lateralis of severe COPD patients, with either normal or low muscle mass, protein levels of TNF-α, its soluble receptor II, and VEGF were reduced consistently compared with the controls.

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**Fig. 4.** A: representative examples of muscle fibers positively stained for active caspase-3 (×400, light microscopy) in the diaphragm of a severe COPD patient. A fiber was considered to be positively stained for active caspase-3 when a nucleus and its surrounding cytoplasm was stained for anti-caspase-3 antibody. Note that the no. of caspase-3-positive nuclei is extremely low in the muscle cross section. B: representative examples of muscle fibers positively stained for active caspase-3 (×400, light microscopy) in the vastus lateralis of the same severe COPD patient. A fiber was considered to be positively stained for active caspase-3 when a nucleus and its surrounding cytoplasm was stained for anti-caspase-3 antibody. Note that the no. of caspase-3-positive nuclei is extremely low in the muscle cross section.

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**Fig. 5.** A: representative examples of immunoblots corresponding to the detection of the proteolytic fragments of caspase-3: 17-kDa active caspase-3 and 35-kDa precursor caspase-3 in both diaphragm and vastus lateralis muscles of severe and moderate COPD patients and control subjects. B: mean values and SD of the percentage of the ratio of cleaved caspase-3 to procaspase-3 in both diaphragm (left) and vastus lateralis (right) muscles are depicted. No significant differences (ns) were detected in the levels of this marker between severe (n = 10) and moderate (n = 8) COPD patients and control subjects (n = 10) in either diaphragm or vastus lateralis muscles. MW, mol wt.
In the current investigation, the number of inflammatory cells was very low in both respiratory and limb muscles from all COPD patients and control subjects. Nevertheless, it should be noted that there was a statistically significant increase in the inflammatory cell numbers in the vastus lateralis of the severe patients. This significant increase, however, is likely to be of little biological relevance, since absolute levels were extremely low in all muscles. Clearly, these results are in agreement with three previously published studies demonstrating very low levels of intramuscular inflammatory cells in limb muscles of severe COPD patients (8, 19, 21). Results from these studies (8, 19, 21) and current findings, however, differ from those previously reported by Montes de Oca et al. (26). One likely explanation to account for this discrepancy could be related to the fact that inflammatory cell counts were only performed in four patients in that study and not in the whole population of COPD patients (26).

The present study is the first to report data on the levels of intramuscular inflammatory cells in the respiratory muscles, the diaphragm and the external intercostals, of patients with COPD. Interestingly, we showed in a previous study (13) that mRNA and protein levels of TNF-α and IL-6 were significantly greater in the external intercostals of patients with severe COPD and normal weight than in healthy controls. Although levels of intramuscular inflammatory cells were not explored in that study, muscle mRNA levels of the CD18 panleukocyte marker did not differ between patients and controls (13). This finding is consistent with the findings reported in the present investigation. Taking all this together, it is possible to conclude that intramuscular inflammatory cells probably play no significant role in the reported upregulation of cytokine levels within the respiratory muscles of COPD patients with normal weight (13). This raises the important question that, in COPD, cytokine production is likely to be regulated differentially in respiratory and limb muscles, probably as a result of the differences in activity and function performed by each type of muscle (11, 14). Moreover, this also suggests that the myocytes probably play a crucial role in the production and regulation of these cytokines as previously shown in other models (20). Clearly, future studies will shed light on these important biological mechanisms in skeletal muscles in COPD.

Among several contributors, decreased proportions of type I fibers in the vastus lateralis have been observed previously in association with peripheral muscle dysfunction of patients with COPD (2). Consistent with previous studies, in the present investigation, quadriiceps muscle function and proportions of type I fibers were reduced significantly in both moderate and severe COPD patients compared with controls (2, 10, 19, 42). It should also be underscored that the population of COPD patients, both moderate and severe, included in the current study exhibited decreased muscle strength without significant alterations in their body composition. In keeping with previous studies (10, 19, 38), severe COPD patients with relatively normal body composition were also shown to exhibit reduced muscle strength. In fact, mechanisms such as hypoxia, medication, deconditioning, oxidative stress, and muscle structure alterations are also proposed contributors to the COPD muscle dysfunction irrespective of the patients’ body composition (2, 4–6, 38). Furthermore, in the current investigation, severe COPD patients also exhibited reduced respiratory muscle strength together with a diaphragm fiber type shift toward a more fatigue-resistant phenotype (increased proportions of slow-twitch muscle fibers), which has also been repeatedly shown and discussed in previously published studies (4, 14, 22–24).

**Muscle Fiber Apoptosis**

In the current investigation, compared with control subjects, levels of TUNEL-positive nuclei were significantly greater in the diaphragm of both moderate and severe COPD patients compared with the controls. Because the TUNEL assay may also detect DNA breaks that are characteristic of active DNA repair activity and hypertrophy, it can be argued that the diaphragm muscle, which must remain active throughout the existence of the individual, is likely to be exposed to a continuous repair process under physiological conditions. On this basis, it could be considered that, in multinucleated muscle fibers, signs of apoptosis rather represent an underlying remodeling process of nuclear domains (14).

The vastus lateralis muscles of the severe patients also exhibited a significant rise in TUNEL-positive nuclei, which, in turn, were inversely correlated with quadriiceps muscle strength. Importantly, the vastus lateralis of the severe COPD patients exhibited similar levels of TUNEL-positive nuclei to those detected in the diaphragms of the same patients. This leads to the conclusion that limb muscles of severe COPD patients with preserved body composition are also likely to be exposed to a continuous repair/remodeling process as indicated by their greater amounts of TUNEL-positive nuclei. Additionally, the fact that patients with lower QMVC were also those exhibiting greater levels of TUNEL-positive nuclei in their lower limb muscles also supports such a conclusion. In line with this, muscle injury and remodeling were clearly shown to occur in muscles of severe COPD patients with normal weight and no other comorbid conditions (12, 13, 25, 28). Indeed, it is likely that, for the muscle fibers to adapt to exercise training or other circumstances, a process of muscle damage and repair should take place within the fibers (12, 13, 25, 28).

It could be anticipated that, in COPD patients with severe muscle wasting, muscle remodeling may probably occur to a much lesser extent than in patients with preserved body composition. In this regard, the present investigation focused its attention on the study of COPD patients exhibiting normal body composition, since new therapeutic strategies may still be offered to this specific population. A second reason for focusing on the study of COPD patients with preserved muscle mass was to unravel discrepancies in the literature with regard to cellular inflammation and apoptosis in the limb muscles of this type of patients (1, 10, 17, 21, 26). Finally, it should also be mentioned that, in our geographical area, the prevalence of nutritional abnormalities among COPD patients is much lower than in other regions (15), thus making this population an interesting target for research.

Agustí et al. (1) also showed in a previous study that severe COPD patients with normal weight also exhibited increased levels of TUNEL-positive nuclei in their vastus lateralis compared with control subjects. Importantly, in that investigation, severe COPD patients with low BMI exhibited even much greater levels of DNA fragmentation (TUNEL assay) in their vastus lateralis compared with healthy controls and severe...
COPD patients with normal weight. The authors concluded that levels of muscle apoptosis were very high in severe COPD patients with low BMI and that these levels inversely correlated with the patients’ exercise capacity (1). In their study (1), levels of poly-(ADP-ribose)-polymerase proteolytic fragments, another marker of apoptosis, were also shown to be increased considerably in the limb muscles of severe COPD with reduced body weight. Ultrastructural analysis of myonuclear apoptosis or other markers, however, was not explored in that study (1).

In the current investigation, the diaphragm and vastus lateralis muscles exhibited extremely few fibers positively stained for active caspase-3 in both severe COPD patients and control individuals. Also, the ratio of cleaved caspase-3 to pro-caspase-3 was low in the respiratory and limb muscles of both patients and controls. These findings are in complete agreement with those reported in a previous study (19) in which no cleaved caspase-3 immunohistochemical localization was found in the limb muscles of either COPD patients or control subjects.

It has been well established that ultrastructural evaluation of cells is the gold standard for the diagnosis of apoptosis. Electron microscopy solely contributes to the diagnosis of apoptosis and confirms the diagnosis made by other indirect methodologies (immunohistochemical procedures on light microscopy). Furthermore, early phases of apoptosis can only be identified using electron microscopy, whereas late phases of this phenomenon are seen on both light and electron microscopy. However, electron microscopy has been so far not widely used for this purpose, probably because of difficulties with its availability. To the best of our knowledge, the present study is the first to report data on the ultrastructural diagnosis of apoptotic nuclei in both respiratory and limb muscles of patients with severe COPD. Importantly, diaphragm and vastus lateralis muscles exhibited low and similar levels of either early or advanced ultrastructural nuclear apoptosis in both severe COPD patients and control subjects. These findings clearly confirm results obtained through the immunohistochemical and immunoblotting techniques (caspase-3) in the current investigation and enable us to conclude that nuclear apoptosis in both respiratory and limb muscles of severe COPD with preserved body composition, at least beyond the “physiological” levels encountered in the muscles of the control subjects.

Study Critique

A first limitation in this investigation is related to the relatively lower number of diaphragm muscle specimens analyzed (inflammation studies) compared with the vastus lateralis and external intercostal muscles (see additional information in the online supplement). However, diagnostic-therapeutic thoracotomy is the only approach available for studying the diaphragm muscle of COPD patients and control subjects. A second limitation has to do with the fact that apoptotic analyses were not conducted in the external intercostal muscles. For ethical reasons, sample specimens obtained from the external intercostals were of smaller size than those obtained from the vastus lateralis and diaphragm muscles. Therefore, no “extra” fragment could be fixated in glutaraldehyde to conduct the ultrastructural analyses on these muscles in any study subject. Furthermore, because the procedures chosen for the quantification of apoptotic nuclei in this study are extremely laborious, especially the electron microscopy analyses, we rather chose to explore all of the apoptotic markers in the main inspiratory muscle, the diaphragm, of those patients bearing a more severe disease.

A third limitation refers to the correlation analyses performed between the cellular findings encountered in the diaphragm and intercostals and the muscle function parameter MIP. In this regard, it should be acknowledged that not only intrinsic muscle abnormalities contribute to respiratory muscle weakness in COPD patients, since other factors such as abnormal muscle configuration and length-tension relationships are also strongly involved.

In conclusion, in severe COPD patients with preserved body composition, while increased apoptotic nuclei seems to be a contributor to their skeletal muscle dysfunction, cellular inflammation does not. The increased numbers of TUNEL-positive nuclei in their muscles suggest that they may also be exposed to a continuous repair/remodeling process.

ACKNOWLEDGMENTS

We are thankful to Dr. M. L. Blanco for help with the patient recruitment and to Miguel A. Martinez-Gonzalez and Luis Magan for technical support.

REFERENCES


