Inflammatory Cytokines and Repair Factors in the Intercostal Muscles of Patients With Severe COPD

Carme Casadevall, Carlos Coronell, Pilar Ausín, Juana Martínez-Llorens, Mauricio Orozco-Levi, Esther Barreiro, and Joaquim Gea* on behalf of the ENIGMA Group in COPD

Objective: There is disagreement regarding the local action of cytokines in the respiratory muscles of patients with chronic obstructive pulmonary disease (COPD). The objective of this study was to analyze the relationships between cytokine expression and genetic activation of the mechanisms of muscle repair.

Patients and Methods: Twenty-five patients with severe COPD and in stable condition were enrolled in the study. We performed a biopsy of the external intercostal muscle of the patients and analyzed the specimen for signs of muscle lesion (morphometry), infiltration of inflammatory cells (immunohistochemistry), and expression of selected genes (real-time polymerase chain reaction technique) corresponding to the cytokines (tumor necrosis factor α [TNF-α] and its type 1 and 2 receptors [TNFR1 and TNFR2], and interleukin [IL] 1β, 6, and 10), a pan-leukocyte marker (CD18), and key molecules in the repair-myogenesis pathways (Pax7, M-cadherin, and MyoD).

Results: Expression of TNFR2 is directly related to inspiratory muscle function (represented by maximum sustainable inspiratory pressure; \( r=0.496, P<.05 \)), whereas expression of CD18 is inversely related (\( r=0.462, P<.05 \)). Moreover, expression of the 2 TNF-α receptors was directly related to that of the key molecules of the repair pathways analyzed (TNFR1 to Pax7 \( r=0.650; P<.001 \) and M-cadherin \( r=0.678; P<.001 \); TNFR2 to Pax7 \( r=0.395; P<.05 \), M-cadherin \( r=0.409; P<.05 \), and MyoD \( r=0.418; P<.05 \)).

Conclusions: Expression of TNF-α receptors bears a close relationship both to activation of the myogenesis programs and to inspiratory muscle function. This reinforces our hypothesis that some local cytokines take part in the repair of respiratory muscles in patients with COPD.

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Introduction

The respiratory muscles of patients with chronic obstructive pulmonary disease (COPD) are subject to an increased workload due to the mechanical changes that take place in the respiratory system. Furthermore, these changes result in a number of structural and functional changes in the muscles themselves. Muscle force and strength are also reduced, which is known as muscle dysfunction, and the muscle therefore becomes more sensitive to mechanical failure. Cell damage may also occur, accompanied by inflammatory and repair elements. It seems clear that remodeling of the muscle takes place, with changes in the fiber phenotype and in other muscle components.\(^1\) Our group recently reported an increase in local concentrations of specific proinflammatory cytokines in the external intercostal muscles and diaphragm in patients with COPD.\(^2,3\) The role of these substances and their relationship with other process, however, remains unknown.\(^2,4\) Experimental evidence shows that some cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6), may favor proteolysis and fiber disruption, as well as directly affecting contractile changes.\(^5,6\) Nevertheless, it has also been found that synthesis of these cytokines and their receptors increases after increasing the muscle workload and/or inducing muscle injury,\(^7,8\) and that blocking this synthesis prevents correct repair.\(^9-12\) Muscle repair depends on the formation of new muscle (myogenesis), which, in adult vertebrates, takes place by means of the activation of satellite cells, which are in turn regulated by the genes of selected cytokines and their receptors play a role in muscle repair in patients with COPD; a local increase in production of these cytokines would therefore be beneficial.\(^1\) Hence, the objective of this study was to evaluate the expression in muscle of the genes of selected cytokines and their relationship with cell damage and with the expression of myogenic factors (repair programs) in these patients.

Patients and Methods

Patients

The sample size was calculated based on previous studies by our group.\(^3,4\) We included 25 patients with stable severe COPD, who had visited the outpatient clinics of our department. The diagnosis of severe–very severe COPD was based on the criteria of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (forced expiratory volume in 1 second/or forced vital capacity <70%, forced expiratory volume in 1 second <50% of reference values, with a negative standard bronchodilation test).\(^3,4\) Stability was defined as the absence of changes in semiology and/or medication in the 3 months prior to the study. To avoid possible interference from associated factors, we excluded patients with chronic respiratory insufficiency (PaO\(_2\) < 60 mmHg at rest), malnutrition (body mass index <20 kg/m\(^2\) and/or body mass index <18 kg/m\(^2\), cardiovascular or neuromuscular problems, treatment with drugs that might affect muscle structure or function, and patients included in rehabilitation programs or with restricted mobility. The cross-sectional study was designed in accordance with the principles of the World Medical Association and approved by the ethics committee of our institution. All patients signed an informed consent in order to take part in the study.

Muscle samples were obtained in accordance with the technique described in detail in previous studies.\(^3,4\) Following local anesthesia with lidocaine, 2 cm a horizontal incision was made at the 6th intercostal space and anterior axillary line, below the lower limit of the pectoral muscle. The sample was extracted using scissors, parallel to the fibers, and sutured by planes. A portion of the sample was quickly frozen in liquid nitrogen and stored at −70°C; the other portion of the sample was preserved in paraffin.

Intercostal Muscle Biopsies

Muscle force was evaluated by measuring maximum inspiratory pressure in a static maneuver from tidal volume and using reference values for the local population.\(^8\) Resistance was measured by determining the maximum sustainable inspiratory pressure (MSIP) and the length of time for which a submaximum load could be sustained; for this purpose, we used the threshold load test according to the method described in detail in previous studies.\(^3,4\) In the first part of the test, patients breathed with threshold incremental inspiratory resistances (8 cm H\(_2\)O every 2 min) until failure. The MSIP was established as the maximum pressure attained. In the second part of the test, patients breathed with a sustained submaximum load equivalent to 80% of the MSIP, also until failure. The time for which the breathing effort was maintained was defined as the sustained breathing time with a submaximum load.

Assessment of Cell Damage

The paraffin-embedded sample was cut into 3-µ sections and stained with hematoxylin–eosin. The proportion of abnormal muscle fibers was quantified by means of optical microscopy (Olympus BX61, Olympus Life and Material Science Europe GmbH, Hamburg, Germany) and the proportion of abnormal muscle fibers was calculated by using the following formula: proportion of abnormal muscle fibers = (number of abnormal muscle fibers per 100 muscle fibers) × (100 × ratio of abnormal muscle fibers). The proportion of abnormal muscle fibers was calculated by using the following formula: proportion of abnormal muscle fibers = (number of abnormal muscle fibers per 100 muscle fibers) × (100 × ratio of abnormal muscle fibers). The proportion of abnormal muscle fibers was calculated by using the following formula: proportion of abnormal muscle fibers = (number of abnormal muscle fibers per 100 muscle fibers) × (100 × ratio of abnormal muscle fibers).
Germany) using the grid counting system adapted for paraffin-embedded samples. The area of abnormal muscle, considered to be a good indicator of the degree of structural damage, was defined as the percentage of points in which the standard criteria of structural alteration was identified, with respect to the total points evaluated. These criteria were internalized nuclei, detectable interstitial nuclei, lipofuscin, small fibers with oblique angles or basophilic cytoplasm, fibers containing necrotic matter, and vessels. Normal reference values were taken from the literature.

**Expression of the Selected Genes**

The real-time polymerase chain reaction technique was used due to its level of quantitative accuracy. The following 12 genes were evaluated by determining their transcriptomes (messenger RNA) in the muscle:

- Cytokines: TNF-α and its 2 receptors (TNFR1 and TNFR2), IL-1β, IL-6, and IL-10.
- Leucocyte marker: CD18 pan-leukocyte integrin.
- Cell damage or stress markers: non-adult heavy chain myosin isoforms—both embryonic (MyHC-emb) and perinatal (MyHC-peri).
- Genes associated with myogenesis: Pax7, M-Cadherin, and Myo-D.

Details regarding performance of the technique are described in another study: The RNA was extracted (RTZipol method, Life Technologies, Frederick, MD, USA) and the complementary DNA was then synthesized (GeneAmp PCR system 2400, Perkin Elmer, Richmond, CA, USA). The PCR reactions were performed after reverse transcription (ABI PRISM 7900HT sequence detection system and TaqMan analysis [Assays-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA, USA]) (Table 1). All samples were processed in triplicate. As an endogenous control gene (housekeeping), we used β2-microglobulin due to its considerable stability in muscle. Data were analyzed with the Sequence Detector software program, version 2.1. (SDS 2.1), using the standard comparative method for relative quantification (Ct).

**Statistical Analysis**

Data are expressed as mean (SD). The degree of relationship between the quantitative variables was evaluated by means of the Pearson product moment correlation. Statistical significance was established for P = .05.

**Results**

**Clinical and Functional Characteristics**

The characteristics of the patients and their nutritional status, lung function and inspiratory muscle function data are shown in Table 2. Patients’ anthropometry and body composition was normal; lung function showed considerable obstruction of the airways, lung hyperinflation, reduced carbon monoxide transfer, and mild hypoxemia at rest, with no hypercapnia. Inspiratory muscle function was abnormal, with reduced force and strength.

**Muscle Damage and Presence of Inflammatory Cells**

Signs of damage were slight (mean [SD], 1.38% [1.09%] abnormal muscle), essentially due to the presence of internalized nuclei. The number of inflammatory cells was also relatively low (2.38 [2.32] mm²), with a slight inverse relationship with inspiratory muscle function. This relationship was significant for expression of the CD18 pan-leukocyte genetic marker in both the force and strength of the inspiratory muscles (Figure 1a).

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Test Identification</th>
<th>Probe Sequence (5'-3')</th>
<th>GenBank Access</th>
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</thead>
<tbody>
<tr>
<td>Cytokines and receptors</td>
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<tr>
<td>IL-1β</td>
<td>Hs00174097_m1</td>
<td>TATGCGAACACAGCTGTGTTCC</td>
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<td>IL-6</td>
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<td>IL-10</td>
<td>Hs00174086_m1</td>
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<td>CTTGTGCTGCACTGGTGTCCTCGTG</td>
<td>NM_001065</td>
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<tr>
<td>Myogenesis-repair</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pax7</td>
<td>Hs00242962_m1</td>
<td>CTGCCGCAACAAGGACAGGCCGCTG</td>
<td>NM_002584</td>
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<td>M-Cadherin</td>
<td>Hs00170504_m1</td>
<td>CATGTGTCCTCAAGGACAGGCC</td>
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<td>Myo-D</td>
<td>Hs00159592_m1</td>
<td>GCCGCCAGCGACAGGGGCCC</td>
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<td>Cell damage/stress</td>
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<td>MyHC-emb</td>
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<td>MyHC-peri</td>
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<td>GATCTGCAAGAGCAAGAAGACCTT</td>
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<td>Pan-leukocyte marker</td>
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<td>CD18</td>
<td>Hs001015742_m1</td>
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<td>Endogenous control (housekeeping)</td>
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<td>Hs0019999907_m1</td>
<td>AGTGGGATCGAGACATGTAAGCAG</td>
<td>NM_004048</td>
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</tbody>
</table>

Abbreviations: CD18, pan-leukocyte integrin; MyHC-emb, embryonic myosin heavy chain; MyHC-peri, perinatal myosin heavy chain; Pax7, paired box gene; TNF-α, tumor necrosis factor-α; TNFR1 and TNFR2, TNF-α receptors 1 and 2, respectively.
Expression of Genes Associated With Cytokines and Myogenesis

Expression of the different inflammatory cytokines showed no significant association with the functional variables. The receptor TNFR2, however, was directly related to inspiratory muscle strength (Figure 1b). Expression of the 2 TNF-α receptors was directly related with each other (Table 3) and with the expression of several other selected molecules of the repair/myogenesis programs (Figure 2 and Table 3).

Expression of TNF-α was directly related to both general damage (Figure 3a and Table 3) and to the specific presence of intracellular nuclei (r=0.575, P<.01). A direct relationship was also observed between the expression of genetic markers for damage (Table 3), both with each other (MyHC-emb and MyHC-peri) and with markers for regeneration (eg, between M-cadherin and both MyHC-emb and MyHC-peri). These regeneration markers also showed a direct internal relationship (eg, M-cadherin and both Pax7 and Myo-D) (Figure 3b and Table 3).

Discussion

The essential finding of this study is the existence of the close relationships observed in the expression of 2 groups of genes with apparently different functions (those associated with cytokine activity and those associated with myogenesis/repair programs) in the respiratory muscles of patients with COPD. Furthermore, the study confirms damage to the muscle structure and the relative absence of inflammatory cells.

The observation of a small percentage of muscle with signs of damage20,21 supports previous findings by our group. In these previous studies, in which other histologic techniques were used, we also observed structural abnormalities in the different respiratory muscles of patients with COPD.22 The results of this study also support our previous observations in animal models, according to which, increased respiratory load may cause muscle damage.23

The study also expands on previous findings by our group regarding the presence of cytokines in the muscles. Expression of these substances and their receptors, in the absence of a significant number of inflammatory cells20,22,24 indicates a probable essentially muscular origin. It is now clear that the fibers are capable of synthesizing different cytokines, which would act as an autocrine/paracrine mechanism.20,22 Nevertheless, synthesis by other cell types, such as blood cells, cannot be ruled out. The role of cytokines in the muscles is still unclear. We know that they promote the loss of proteins and have a direct harmful effect on contraction.25,26 Furthermore, their appearance following intense exercise and/or muscle damage27 suggests that they play a relevant role in repair. Similarly, recent studies have shown that the absence of TNF-α receptors determine repair of defective muscle,10,11 as their activation is essential for myogenic differentiation. Our results, which show a close association between the expression of TNF-α receptors and myogenic factors in the intercostal muscles of patients with COPD, appear to point in this direction. A similar interpretation may be applied to the direct relation observed between the expression of these receptors and muscle function.28,29 All of this suggests that local cytokines play a relevant role in the repair and conservation of muscle function.

The mechanism of stimulation of cytokine synthesis in the respiratory muscles is still unclear, but it probably depends on prior activity, cell damage, and/or apoptosis.7,13,14,30 Overexpression of these substances has been observed in the diaphragm of rats subjected to increased respiratory loads31 or experimental emphysema.32

Limitations of this study include the fact that it focused on examining the relationships between different biologic phenomena in patients with COPD. Comparison with a control group was not performed for 2 basic reasons. First, because the specific objective of the study was to examine the relationships between muscle damage, cytokine expression, and the activation of repair programs in the

Table 2

<table>
<thead>
<tr>
<th>General data and nutritional assessment</th>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>67 (6)</td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.4 (3.9)</td>
<td>19.8 (1.3)</td>
</tr>
<tr>
<td>Lung function</td>
<td></td>
<td></td>
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<tr>
<td>FEV₁, % of reference</td>
<td>31 (10)</td>
<td></td>
</tr>
<tr>
<td>FEV₁/FVC, %</td>
<td>43 (9)</td>
<td></td>
</tr>
<tr>
<td>RV/TLC, %</td>
<td>65 (10)</td>
<td></td>
</tr>
<tr>
<td>DLCO, % of reference</td>
<td>57 (16)</td>
<td></td>
</tr>
<tr>
<td>PaO₂, mm Hg</td>
<td>72 (9)</td>
<td></td>
</tr>
<tr>
<td>PaCO₂, mm Hg</td>
<td>42.2 (3.1)</td>
<td></td>
</tr>
<tr>
<td>Inspiratory muscle function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP, % of reference</td>
<td>61 (21)</td>
<td></td>
</tr>
<tr>
<td>MSIP, cm H₂O</td>
<td>–45 (13) (NV &gt;–55)</td>
<td></td>
</tr>
<tr>
<td>TLim, min</td>
<td>12.4 (6.6) (NV &gt;15)</td>
<td></td>
</tr>
</tbody>
</table>

* Data are presented as mean (SD). Abbreviations: BMI, body mass index; DLCO, carbon monoxide transfer; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; MIP, maximum inspiratory pressure; MSIP, maximum sustainable inspiratory pressure; NV, normal values in our laboratory; RV, residual volume; TLC, total lung capacity; TLim, sustained inspiratory time.

![Figure 1](http://www.archbronconeumol.org)

**Figure 1.** a) Relationships between force, represented by maximum inspiratory pressure (MIP), and strength, expressed by sustainable MIP (MSIP), of the inspiratory muscles, and expression of the CD18 leukocyte marker, and b) relationship between muscle strength and expression of tumor necrosis factor α receptor 2 (TNFR2). Abbreviation: au, arbitrary units.
Table 3
Correlations Between the Different Biological Variables

<table>
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<tr>
<th></th>
<th>TNFR1, au</th>
<th>TNFR2, au</th>
<th>IL-1β, au</th>
<th>IL-6, au</th>
<th>Abnormal Muscle, %</th>
<th>MyHC-emb, au</th>
<th>MyHC-peri, au</th>
<th>Pax7, au</th>
<th>M-Cadherin, au</th>
<th>Myo-D, au</th>
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<tbody>
<tr>
<td>TNF-α, au</td>
<td>r = -0.049</td>
<td>r = 0.356</td>
<td>r = -0.12</td>
<td>r = -0.13</td>
<td>r = 0.518</td>
<td>r = 0.189</td>
<td>r = 0.192</td>
<td>r = 0.180</td>
<td>r = 0.121</td>
<td>r = 0.104</td>
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<tr>
<td>TNFR1, au</td>
<td>r = 0.456</td>
<td>r = 0.147</td>
<td>r = 0.054</td>
<td>r = 0.139</td>
<td>r = 0.04</td>
<td>r = 0.033</td>
<td>r = 0.15</td>
<td>r = 0.05</td>
<td>r = 0.11</td>
<td>r = 0.032</td>
</tr>
<tr>
<td>TNFR2, au</td>
<td>P &lt; 0.05</td>
<td>P = 0.62</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.15</td>
<td>P &gt; 0.85</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>IL-1β, au</td>
<td>P &lt; 0.05</td>
<td>P &gt; 0.00</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
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<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>IL-6, au</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
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</table>

Abbreviations: au, arbitrary units; IL, interleukin; MyHC-emb, embryonic myosin heavy chain; MyHC-peri, perinatal myosin heavy chain; Pax7, paired box gene; TNF-α, tumor necrosis factor-α; TNFR1 and TNFR2, TNF-α receptors 1 and 2, respectively.

Figure 2. Direct relationships observed between expression of the 2 receptors of tumor necrosis factor-α (TFFR1 and TNFR2) and the different molecules associated with repair/myogenesis programs (Pax7, M-cadherin, and Myo-D). Abbreviations: au, arbitrary units; NS, not significant.

muscles of the patients. This, together with the relative aggressiveness of the procedure, would not justify including healthy subjects. Second, because our group has already published studies in which comparison was carried out with a control group and we have obtained values that can be used as a reference. Additionally, in healthy subjects in normal circumstances, muscle repair programs remain relatively inactive.

Furthermore, we decided to examine the external intercostal muscle, which is not considered to be the main inspiratory muscle, due to our intention to exclude any comorbidity. We know that diaphragm samples are usually obtained from patients undergoing surgery due to a severe associated disease.

Finally, the technique used to evaluate the expression of the genes (real-time PCR) does not make it possible to guarantee their
cellular origin. Nevertheless, this is the most suitable technique for the principal objective of this study, which was to provide a quantitative evaluation of the expression of these genes.

In summary, this study confirms the presence of cell damage, though scarce, in the external intercostal muscle of patients with COPD, while also showing low concentrations of inflammatory cells and a close association between the expression of TNF-α receptors and the activation of myogenesis programs. This last finding suggests that this cytokine plays a relevant role in the repair and remodeling of respiratory muscles in patients with COPD.

Funding

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31. Wang X, Jiang TX, Road JD, Redenbach DM, Reid WD. Granocytosis and increased adhesion molecules after resistive loading of the diaphragm. Eur Respir J. 2005;26:786-94.
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