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Original Article

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

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ABSTRACT

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, IL-6, and IL-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.

Citocinas inflamatorias y factores de reparación en los músculos intercostales de pacientes con EPOC grave

Palabras clave:
Daño celular
Miocinas
Inflamación
Reparación
Músculos respiratorios

RESUMEN

Introducción: Las acciones locales de las citocinas en los músculos de los pacientes con enfermedad pulmonar obstructiva crónica (EPOC) se hallan sometidas a debate. El objetivo del presente estudio ha sido analizar las relaciones entre su expresión y la activación genética de programas de reparación muscular.
Pacientes y métodos: Se incluyó en el estudio a 25 pacientes con EPOC grave en situación estable. Se les realizó una biopsia del músculo intercostal externo, donde se evaluaron los signos de lesión muscular (morfometría), la infiltración de células inflamatorias (inmunohistoquímica) y la expresión de genes seleccionados (técnica de reacción en cadena de la polimerasa en tiempo real) correspondientes a las propias citocinas –factor de necrosis tumoral alfa (TNF-α) y sus receptores 1 y 2 (TNFR1 y TNFR2), e interleucinas-1β, 6 y 10–, un marcador panleucocitario (CD18) y moléculas clave en las vías de reparación-miogénesis (Pax7, M-Caderina y Mio-D).

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Resultados: La expresión de TNFR2 se relacionó directamente con la función muscular inspiratoria (representada por la presión inspiratoria máxima sostenible; r = 0,496, p < 0,05), mientras que la expresión de CD18 se relacionó inversamente con ella (r = –0,462, p < 0,05). Por otra parte, la expresión de los 2 receptores del TNF-α se relacionó directamente con la de las moléculas clave de las vías de reparación analizadas (TNFR1 con Pax7, r = 0,650, y M-Caderina, r = 0,678, ambas con p < 0,001; TNFR2 con Pax7, r = 0,395, M-Caderina, r = 0,409, y Mio-D, r = 0,418, con p < 0,05 en todas).
Conclusiones. La expresión de los receptores del TNF-α guarda una estrecha relación tanto con la activación de los programas de miogénesis como con la propia función muscular inspiratoria. Este hecho refuerza nuestra hipótesis de que algunas citocinas locales participan en la reparación de los músculos respiratorios en los pacientes con EPOC.

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,[7-9] and that blocking this synthesis prevents correct repair.[10-12] 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 signal pathways, involving paired-box 7 (Pax7), M-Cadherin, and MyoD, together with other myogenic regulation factors.[13] Our hypothesis is that certain 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.[3] 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.[2,4] 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/forced vital capacity <70%, forced expiratory volume in 1 second <50% of reference values, with a negative standard bronchodilation test).[14] 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 mm Hg 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.

Lung Function Testing and Nutritional Study. We performed forced spirometry with bronchodilation, determination of lung volumes and airway resistance (body plethysmography), CO transfer measurements, and arterial blood gas analysis, in accordance with standard techniques and using reference values appropriate for the local population.[15-17] Nutritional assessment included calculation of the body mass index (anthropometry) and the lean mass index (electric bioimpedance).

Inspiratory Muscle Function. We evaluated the force and strength of the respiratory muscles. Muscle force was evaluated by measuring maximum inspiratory pressure in a static maneuver from tidal volume and using reference values for the local population.[18] 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.[19] In the first part of the test, patients breathed with threshold incremental inspiratory resistances (8 cm H_2O 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.

Intercostal Muscle Biopsies

Muscle samples were obtained in accordance with the technique described in detail in previous studies.[19] 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.

Assessment of Cell Damage

The paraffin-embedded sample was cut into 3-μ sections and stained with hematoxylin–eosin The proportion of abnormal muscle was determined by means of optical microscopy (Olympus BX61, Olympus Life and Material Science Europe GmbH, Hamburg,

Germany) using the grid counting system[20] 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.[20] Normal reference values were taken from the literature.[20-22]

Presence of Inflammatory Cells

New 3 μm sections were taken from the paraffin-embedded samples and subjected to standard immunohistochemical procedures using monoclonal antibodies specific for inflammatory cells: anti-CD45 (generic leukocyte marker) and anti-CD68 (specific marker for monocytes/macrophages), PG-M1 2B11 clones, and PD7/26 (Dako Cytomation Inc, Carpentaria, CA, USA). The sections were mounted on slides pretreated with Poly-L-lysin and then deparaffinized and rehydrated. Reactivity to the primary antibodies was detected using the traditional avidin-biotin-immunoperoxidase method (LSAB+HRP, Dako Cytomation Inc, Carpentaria, CA, USA). The primary antibody was omitted in the negative controls. Positive results for CD45 and/or CD68, determined using optical microscopy with digital imaging, is expressed in the form of positive cells per square millimeter. Normal reference values were taken from the literature.[20,22-24]

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.[2] The RNA was extracted (RTIzol 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 β_2-microglobulin due to its considerable stability in muscle.[25] Data were analyzed with the Sequence Detector software program, version 2.1. (SDS 2.1), using the standard comparative method for relative quantification (C_T).[2,26]

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] mm^2), 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
Sequence of Probes Used in Each Case for the Real-Time Polymerase Chain Reaction Technique

Gene	Test Identification	Probe Sequence (5?-3?)	GenBank Access
Cytokines and receptors			
IL-1β	Hs00174097_m1	TATGGAGCAACAAGTGGTGTTCTCC	NM_000576
IL-6	Hs00174131_m1	ATTCAATGAGGAGACTTGCCTGGTG	NM_000600
IL-10	Hs00174086_m1	GCCTTTAATAAGCTCCAAGAGAAAG	NM_000572
TNF-α	Hs00174128_m1	ATGTTGTAGCAAACCCTCAAGCTGA	NM_000594
TNFR1	Hs00533560_m1	CCTGCTGCTGCCACTGGTGCTCCTG	NM_001065
TNFR2	Hs00153550_m1	GAAGCCAAGGTGCCTCACTTGCCTG	NM_001066
Myogenesis-repair			
Pax7	Hs00242962_m1	CTGGGCGACAAAGGGAACCGGCTGG	NM_002584
M-Cadherin	Hs00170504_m1	GACTGATCGCTTCAGGCTAAGAGCG	NM_004933
Myo-D	Hs00159528_m1	GGCGCCCAGCGAACCCAGGCCCGGG	NM_002478
Cell damage/stress			
MyHC-emb	Hs00159463_m1	ACAACAGGACCCTGGTGGTCAAACC	NM_002470
MyHC-peri	Hs00267293_m1	GATGTTGCAAAGGAGAGAAGCACTT	NM_002472
Pan-leukocyte marker			
CD18	Hs01051742_m1	GTGGATGAGAGCCGAGAGTGTGTGG	NM-000211
Endogenous control (housekeeping)			
β_2-microglobulin	Hs99999907_m1	AGTGGGATCGAGACATGTAAGCAGC	NM_004048

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 damage[20-22] 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.[2,27] The results of this study also support our previous observations in animal models, according to which, increased respiratory load may cause muscle damage.[28,29]

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 cells[20,22-24] 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.[30-32] 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.[5,6] Furthermore, their appearance following intense exercise and/or muscle damage[7-9] 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.[2,4] 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.[7,9,33,34] Overexpression of these substances has been observed in the diaphragm of rats subjected to increased respiratory loads[9] or experimental emphysema.[34]

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
Patient Characteristics*

General data and nutritional assessment	
Age, y	67 (6)
BMI, kg/m^2	26.4 (3.9)
LMI, kg/m^2	19.8 (1.3)
Lung function	
FEV_1, % of reference	31 (10)
FEV_1/FVC, %	43 (9)
RV/TLC, %	65 (10)
DLCO, % of reference	57 (16)
PaO_2, mm Hg	72 (9)
$PaCO_2$, mm Hg	42.2 (3.1)
Inspiratory muscle function	
MIP, % of reference	61 (21)
MSIP, cm H_2O	−45 (13) (NV >−55)
TLim, min	12.4 (6.6) (NV >15)

*Data are presented as mean (SD). Abbreviations: BMI, body mass index; DLCO, carbon monoxide transfer; FEV_1, 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.

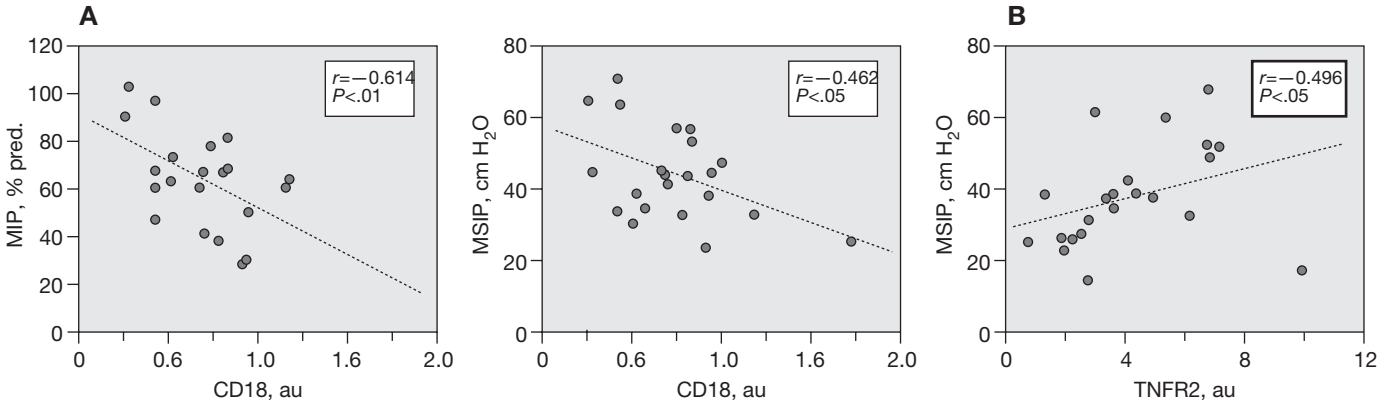

Figure 1. *a*) Relationships between force, represented by maximum inspiratory pressure (MIP), and strength, expressed by sustainable MIP (MSIP), of the inspiratory muscles, and expression of the CD18 leukocyte marker, and *b*) relationship between muscle strength and expression of tumor necrosis factor-α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

Table 3
Correlations Between the Different Biological Variables

	TNFR1, au	TNFR2, au	IL-1β, au	IL-6, au	Abnormal Muscle, %	MyHC-emb, au	MyHC-peri, au	Pax7, au	M-Cadherin, au	Myo-D, au
TNF-α, au	*r*=−0.049 *P*=.815	*r*=0.356 *P*=.08	*r*=−0.12 *P*=.563	*r*=−0.13 *P*= .536	*r*=0.518 *P*=.01	*r*=0.189 *P*=.365	*r*=0.192 *P*=.359	*r*=0.160 *P*=.445	*r*=0.121 *P*=.565	*r*=0.104 *P*=.621
TNFR1, au		*r*=0.456 *P*<.05	*r*=0.147 *P*=.484	*r*=0.054 *P*=.796	*r*=0.139 *P*=.517	*r*=.004 *P*=.986	*r*=0.033 *P*=.876	*r*=0.650 *P*<.001	*r*=0.678 *P*<.001	*r*=0.208 *P*=.318
TNFR2, au			*r*=0.295 *P* =.152	*r*=0.364 *P*=.07	*r*=0.111 *P*=.604	*r*=0.278 *P*=.178	*r*=0.272 *P*=.188	*r*=0.395 *P*=.05	*r*=0.409 *P*<.05	*r*=0.418 *P*<.05
IL-1β, au				*r*=0.559 *P*<.01	*r*=−0.04 *P*=.838	*r*=−0.38 *P*=.06	*r*=−0.18 *P*=.385	*r*=−0.15 *P*=.479	*r*=−0.33 *P*=.107	*r*=−0.09 *P*=.677
IL-6, au					*r*=0.140 *P*=.514	*r*=−0.04 *P*=.857	*r*=−0.09 *P*=.647	*r*=−0.03 *P*=.880	*r*=−0.19 *P*=.364	*r*=−0.09 *P*=.660
Abnormal muscle, %						*r*=0.079 *P*=.715	*r*=0.016 *P*=.942	*r*=0.015 *P*=.944	*r*=0.177 *P*=0.407	*r*=0.299 *P*=0.155
MyHC-emb, au							*r*=0.773 *P*<.001	*r*=0.196 *P*=0.349	*r*=0.402 *P*<.05	*r*=0.138 *P*=0.511
MyHC-peri, au								*r*=−0.04 *P*=0.861	*r*=0.373 *P*=0.06	*r*=0.319 *P*=0.121
Pax7, au									*r*=0.661 *P*<.001	*r*=0.200 *P*=0.338
M-cadherin, au										*r*=0.553 *P*<.01

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.

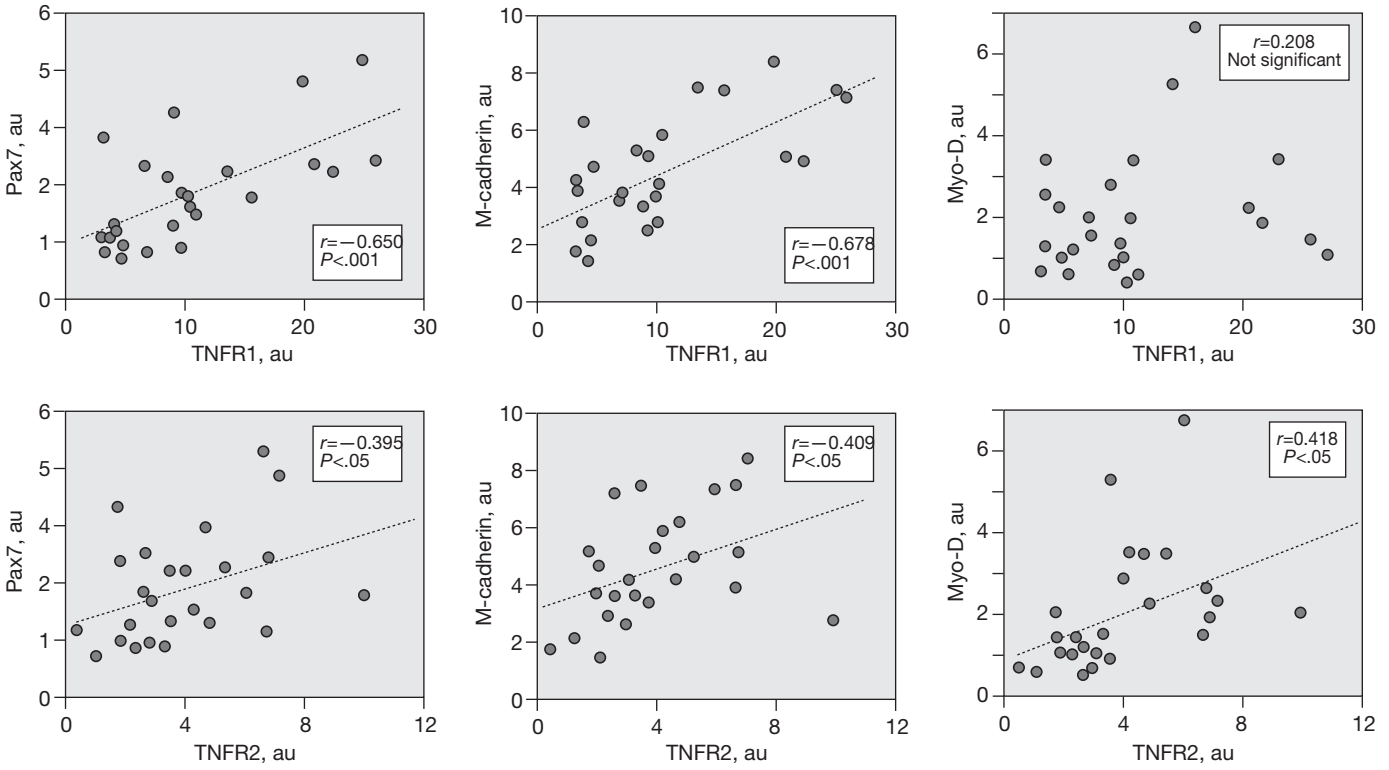

Figure 2. Direct relationships observed between expression of the 2 receptors of tumor necrosis factor-α (TFFR1 and TNFR2) and the different molecules associated with repair/myogenesis programs (Pax7, M-cadherin, and Myo-D). Abbreviations: au, arbitrary units; NS, not significant.

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[2,3] and we have obtained values that can be used as a reference.[20-24] 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

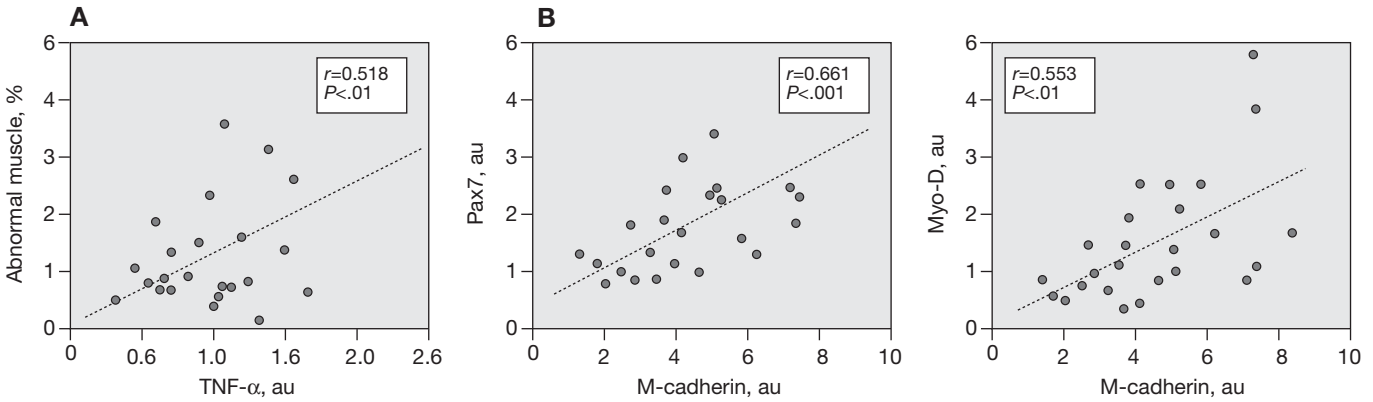

Figure 3. *a)* Relationships between expression of tumor necrosis factor-α (TNF-α) and the proportion of abnormal muscle (muscle damage index), and *b)* internal relationships between different myogenesis activation markers: M-Cadherin with Pax7 and with Myo-D. Abbreviation: au, arbitrary units.

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.

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References

1. Gea J, Barreiro E, Orozco-Levi M. Free radicals, cytokines and respiratory muscles in COPD patients. Clin Pulm Med. 2007;14:117-26.
2. Casadevall C, Coronell C, Ramírez-Sarmiento A, Martínez-Llorens J, Barreiro E, Orozco-Levi M, et al. Upregulation of proinflammatory cytokines in the intercostal muscles of COPD patients. Eur Respir J. 2007;30:1-7.
3. Casadevall C, Barreiro E, Orozco-Levi M, Minguella J, Gea J. Local expression of tumor necrosis factor (TNF)-alpha: is it the baddy or the goody in the story of respiratory muscle adaptation occurring in COPD? [abstract]. Proc Am Thorac Soc. 2006;3:A26.
4. Barreiro E, Schols AMW, Polkey MI, Gáldiz JB, Gosker HR, Swallow EB, et al. Cytokine profile in the quadriceps muscles of patients with severe COPD. Thorax. 2008;63:100-7.
5. Debigare R, Cote CH, Maltais F. Peripheral muscle wasting in chronic obstructive pulmonary disease. Clinical relevance and mechanisms Am J Crit Care Med. 2001; 164:1712-7.
6. Reid MB, Lännergren J, Westerblad H. Respiratory and limb muscle weakness induced by tumor necrosis factor-a. Involvement of muscle myofilaments. Am J Respir Crit Care Med. 2002;166:479-84.
7. Pedersen BK, Ostrowski K, Rohde T, Bruunsgaard H. The cytokine response to strenuous exercise. Can J Physiol Pharmacol. 1998;76:505-11.
8. Tomiya A, Aizawa T, Nagatomi R, Sensui H, Kokubun S. Myofibers express IL-6 after eccentric exercise. Am J Sports Med. 2004;32:503-8.
9. Vassilakopoulos T, Divangahi M, Rallis G, Kishta O, Petrof B, Comtois A, et al. Differential cytokine gene expression in the diaphragm in response to strenuous resistive breathing. Am J Respir Crit Care Med. 2004;170:154-61.
10. Chen SE, Gerken E, Zhang Y, Zhan M, Mohan RK, Li AS, et al. Role of TNF-α signalling in regeneration of cardiotoxin-injured muscle. Am J Physiol Cell Physiol. 2005;289: C1179-C87.
11. Chen SE, Jin B, Li YP. TNF-a regulates myogenesis and muscle regeneration by activating p38 MAPK. Am J Physiol Cell Physiol. 2007;292:C1660-C71.
12. Warren GL, Hulderman T, Jensen N, McKinstry M, Mishra M, Luster MI, et al. Physiological role of tumor necrosis factor alpha in traumatic muscle injury. FASEB J. 2002;16:1630-2.
13. Bryson-Richardson RJ, Currie PD. The genetics of vertebrate myogenesis. Nat Rev Genet. 2008;9:632-46.
14. Global Initiative for Chronic Obstructive Lung Disease, December 2007. Global Strategy for the Diagnosis, Management and Prevention of COPD. Available from: www.goldcopd.com
15. Roca J, Sanchis J, Agustí-Vidal A, Segarra F, Navajas D, Rodríguez-Roisin R, et al. Spirometric reference values from a Mediterranean population. Bull Eur Physiopathol Respir. 1986;22:217-24.
16. Roca J, Burgos F, Barberà JA, Sunyer J, Rodríguez-Roisin R, Castellsague J, et al. Prediction equations for plethysmographic lung volumes. Respir Med. 1998; 92:454-60.
17. Roca J, Rodríguez-Roisin R, Cobo E, Burgos F, Pérez J, Clausen JL. Single-breath carbon monoxide diffusing capacity prediction equations from a Mediterranean population. Am Rev Respir Dis. 1990;141:1026-32.
18. Morales P, Sanchis J, Lamb PJ, Díez JL. Maximum static respiratory pressures in adults. The reference values for a Mediterranean Caucasian population. Arch Bronconeumol. 2007;33:213-9.
19. Ramírez-Sarmiento A, Orozco-Levi M, Güell R, Barreiro E, Hernández N, Mota S, et al. Inspiratory muscle training in patients with chronic obstructive pulmonary disease: structural adaptation and physiologic outcomes. Am J Respir Crit Care Med. 2002;166:1491-7.
20. Macgowan NA, Kenneth GE, Road JD, Reid WD. Diaphragm injury in individuals with airflow obstruction. Am J Respir Crit Care Med. 2001;163:1654-9.
21. Scott A, Wang X, Road JD, Reid WD. Increased injury and intramuscular collagen of the diaphragm in COPD: autopsy observations. Eur Respir J. 2006;27:51-9.
22. Wang X, Jiang TX, Road JD, Redenbach DM, Reid WD. Granulocytosis and increased adhesion molecules after resistive loading of the diaphragm. Eur Respir J. 2005; 26:786-94.
23. Gosker HR, Kubat B, Schaart G, van der Vusse GJ, Wouters EF, Schols AM. Myopathological features in skeletal muscle of patients with chronic obstructive pulmonary disease. Eur Respir J. 2003;22:280-5.
24. Montes de Oca M, Torres SH, de Sanctis J, Mata A, Hernández N, Tálamo C. Skeletal muscle inflammation and nitric oxide in patients with COPD. Eur Respir J. 2005; 26:390-7.
25. Mahoney DJ, Carey K, Fu MH, Snow R, Cameron-Smith D, Parise G, et al. Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. Physiol Genomics. 2004;18:226-31.
26. Livak K, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method. Methods. 2001;25:402-8.
27. Orozco-Levi M, Lloreta J, Minguella J, Serrano S, Broquetas JM, Gea J. Injury of the human diaphragm associated with exertion and chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2001;164:1734-9.
28. Zhu E, Petrof BJ, Gea J, Comtois N, Grassino AE. Diaphragm muscle fiber injury after inspiratory resistive breathing. Am J Respir Crit Care Med. 1997;155: 1110-6.
29. Palacio J, Gáldiz JB, Mariñán M, Orozco-Levi M, Martínez P, Bech JJ, et al. Cellular damage and expression of IL-10 and TNF-α in skeletal muscles following resistive inspiratory breathing [abstract]. Am J Respir Crit Care Med. 2001;163 Suppl:A 147.

30. Saghizadeh M, Ong JM, Garvey T, Henry RR, Kern PA. The expression of TNFα by human muscle. Relationship to insulin resistance. J Clin Invest. 1996;97:1111-6.
31. Li YP, Reid MB. Effect of tumor necrosis factor-alpha on skeletal muscle metabolism. Curr Opin Rheumatol. 2001;13:483-7.
32. Keller P, Keller C, Carey AL, Jauffred S, Fischer CP, Steensberg A, et al. Interleukin-6 production by contracting human skeletal muscle: autocrine regulation by IL-6. Biochem Biophys Res Commun. 2003;310:550-4.
33. Koçtürk S, Kayatekin BM, Resmi H, Açikgöz O, Kaynak C, Ozer E. The apoptotic response to strenuous exercise of the gastrocnemius and solues muscle fibers in rats. Eur J Appl Physiol. 2008;102:515-24.
34. Degens H, Swisher AK, Heijdra YF, Siu PM, Dekhuijzen PN, Alway SE. Apoptosis and Id2 expression in diaphragm and soleus muscle from the emphysematous hamster. Am J Physiol Regul Integr Comp Physiol. 2007;293:R135-R44.