Analysis of Respiratory Muscle Structure and Tumor Necrosis and Insulin-Like Growth Factor Expression in Chronic Obstructive Pulmonary Disease: Are Samples Valid if Obtained During Thoracotomy Performed Because of Localized Pulmonary Neoplasia?

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OBJECTIVE: Various methods have been used to obtain samples to study the structure of human respiratory muscles and the expression of diverse substances in them. Samples are most often obtained from autopsies, from muscle biopsies during thoracotomy performed because of a localized pulmonary lesion (TLL), and from ambulatory thoracoscopic biopsy in patients free of comorbidity (AT). The dis-advantage of the first 2 of these methods lies in the possibility of interference from factors related to the patient's death in the first case or from the disease that necessitated surgery in the second. Although AT is free from the disadvantages of the other 2 methods, it is impossible to obtain samples of the diaphragm—the principal respiratory muscle—with this procedure. The objective of this study was to analyze the fibrous structure of the external intercostal muscle of patients with chronic obstructive pulmonary disease and to quantify the expression of the principal inflammatory cytokine—tumor necrosis factor alpha (TNF-α)—and of insulin-like growth factor (IGF-1) in the same muscle, comparing the results obtained with TLL and AT samples.

METHODS: Prospective and consecutive samples were taken of the external intercostal muscle (fifth space, anterior axillary line) in 15 patients with chronic obstructive pulmonary disease (mean [SD] age 66 [6] years; forced expiratory volume in 1 second 49% [9%] of predicted; PaO_2 75 [9] mm Hg). Samples were taken during TLL (8 patients, all with pulmonary neoplasms but carefully selected in order to rule out systemic effects) or TA (7 patients). Patients with serious comorbidity were excluded from the second group. Samples were processed for structural analysis of fibers (immunohistochemical and enzymatic histochemical) and genetic expression of TNF-α and IGF-1 (real-time polymerase chain reaction).

RESULTS: No differences in the structure of fibers were found between the 2 groups. No differences were observed in the expression of TNF-α or IGF-1.

CONCLUSIONS: Using rigorous criteria, the TLL method appears to be suitable for studying the structural characteristics and expression of inflammatory cytokines and growth factors in the external intercostal muscle. Moreover, it can also be inferred that TLL is probably also useful for obtaining samples of the diaphragm, a muscle which cannot currently be sampled by any alternative method.

Key words: *Respiratory muscles. Fibers. TNF-α. Growth factor. Thoracotomy.*

Análisis estructural y expresión de los factores de necrosis tumoral y crecimiento insulina-*like* en los músculos respiratorios de pacientes con EPOC. ¿Son válidas las muestras obtenidas en el curso de una toracotomía por neoplasia pulmonar localizada?

OBJETIVO: Los estudios estructurales y de expresión de diversas sustancias en músculos respiratorios de seres humanos se han servido de diversos modelos para la obtención de las muestras. Entre ellos destacan la toma de tejidos en autopsias, la biopsia muscular en el curso de una toracotomía por lesión pulmonar localizada (TLL) y la biopsia ambulatoria en sujetos sin comorbilidad (TA). Los 2 primeros modelos adolecen de las posibles interferencias de factores relacionados, respectivamente, con la muerte o la enfermedad que motiva el acto quirúrgico. La TA, aunque obvia los inconvenientes de los otros 2 modelos, no permite obtener muestras del diafragma, principal músculo respiratorio. El

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objetivo de este trabajo fue analizar la estructura fibrilar y expresión de la principal citocina inflamatoria –factor de necrosis tumoral alfa (TNF-α)– y del factor de crecimiento muscular insulina-*like* (IGF-1) en el músculo intercostal externo de pacientes con enfermedad pulmonar obstructiva crónica, comparando los resultados obtenidos con los modelos de TLL y TA.

MÉTODOS: Se tomaron prospectiva y consecutivamente muestras del músculo intercostal externo (quinto espacio, línea axilar anterior) en 15 pacientes con enfermedad pulmonar obstructiva crónica (66 ± 6 años; volumen espiratorio forzado en el primer segundo del 49 ± 9% ref., presión arterial de oxígeno de 75 ± 9 mmHg). Las muestras se tomaron mediante TLL (8 pacientes, todos ellos con neoplasia pulmonar pero cuidadosamente seleccionados para descartar efectos sistémicos) o TA (7 pacientes), excluyéndose en el segundo caso la presencia de comorbilidad importante. Las muestras se procesaron para análisis estructural fibrilar (inmunohistoquímica e histoquímica enzimática) y de expresión génica de TNF-α e IGF-1 (reacción en cadena de la polimerasa en tiempo real).

RESULTADOS: El análisis estructural de las fibras no mostró diferencias entre ambos grupos. Tampoco se observaron diferencias en la expresión de TNF-α o IGF-1.

CONCLUSIONES: Con criterios de selección rigurosos, el modelo de TLL parece adecuado para el estudio de las características estructurales y de expresión de citocinas inflamatorias y factores de crecimiento en el músculo intercostal externo. Puede además inferirse que probablemente la TLL también sea útil para esos objetivos en el caso del diafragma, para el que no existe una técnica alternativa en la actualidad.

Palabras clave: *Músculos respiratorios. Fibras. TNF-α. Factor de crecimiento. Toracotomía.*

Introduction

Chronic obstructive pulmonary disease (COPD) is a highly prevalent entity which places a considerable burden on the health system.[1] Functionally it is characterized by irreversible airway obstruction and hyperinflation of the lung.[2] Hyperinflation causes changes in the geometry of the thorax, the main consequence of which is the elongation of the intercostal muscles and the shortening of the diaphragm. As a result, the muscles evolve away from their optimum contraction length, and this leads to loss of functional capacity.[3] This is compounded by the fact that the muscles have to work with high loads and an abnormal ventilation perfusion ratio, which contribute to the imbalance between the supply and demand of oxygen and nutrients to the muscle. However, in spite of the harmful effects on the respiratory muscle of "external" factors, structural studies have been able to establish that the respiratory muscle also undergoes a process of "intrinsic" adaptation to these conditions.[4,5] These changes are accompanied by an increased ability of muscles to develop strength[6] and, probably, greater resistance in the muscle fibers. The muscles of patients with COPD appear to be subject to other damaging influences, such as oxidative stress[7] and inflammation.[8] Although animal models of exposure to tobacco and of emphysema secondary to physical or chemical aggression allow us to emulate some of the circumstances that cause this disease, such models are probably imperfect with respect to length of exposure and other factors that are present in patients with COPD. All of the above means that it is essential to be able to study the structure and metabolism of the respiratory muscles of actual patients with the disease. The samples needed for such studies can be obtained by various methods. Firstly, tissue samples can be obtained from autopsies.[9] However in this case it is impossible to control for the numerous factors associated with the cause of death. Secondly, samples can be obtained during lung-volume–reduction surgery.[4] This method has become increasingly uncommon in recent years for ethical reasons,[10] and is only a source of samples from seriously ill patients. Thirdly, samples can be obtained from patients undergoing thoracotomy performed because of localized lesions (TLL).[5,11,12] The problem in this case is the likelihood of interference from factors associated with the patient's underlying disease, which is nearly always a neoplastic process. In the fourth place, biopsies can be taken during upper laparotomy.[13] Unfortunately, with the advent of laparoscopic surgery, the indications for this type of intervention have declined drastically. In light of all of the above, the idea was suggested a few years ago of obtaining respiratory muscle samples from the thoracic cage in outpatients (a procedure known as ambulatory thoracoscopic [AT] biopsy). Patients undergoing this procedure can be carefully selected in each case in accordance with the needs of the study design.[14] However, AT sampling is not altogether free of serious disadvantages either, since it is impossible to obtain biopsies of the main respiratory muscle. The most widely used model to date has been TLL, and it is probably appropriate for morphometric studies. However, a reasonable doubt remains that the underlying disease, which, as we have already observed, is usually a neoplastic process, may modify the cellular microenvironment and distort results related to the expression of inflammatory substances or growth factors. Samples of the external intercostal muscle can be obtained with both the TLL and the AT methods (and using the latter it is possible to rule out all comorbidity). The objective of the present study was to examine samples of the external intercostal muscle obtained with TLL and AT, analyzing the genetic expression of the principal inflammatory cytokine (tumor necrosis factor alpha [TNF-α]) and of the principal muscle growth factor (insulin-like growth factor [IGF-1]), as well as muscle fiber structure, and to compare the results obtained with both types of samples.

Methods

Population

A total of 15 patients with COPD[15] were enrolled in the study: 8 with confirmed localized pulmonary neoplasms

waiting for therapeutic thoracotomy and 7 controls. The controls, who were recruited from our hospital's pulmonology outpatient department, were patients with a similar degree of functional impairment but no comorbidity. All the patients studied were individuals who maintained an acceptable degree of physical activity without undertaking specific additional activities, such as regular attendance at a gym or a respiratory rehabilitation program. Furthermore, their condition was stable and they had not had any episodes of decompensation during the 3 months prior to their enrollment in the study. In order to avoid bias caused by hormonal factors, only male patients were enrolled in both groups. In order to avoid other potential sources of bias, the following types of patients were excluded: patients suffering from neuromuscular, endocrine, or cardiovascular diseases, and those with bronchial asthma, chronic respiratory failure (PaO_2 of under 60 mm Hg when stable), or significant malnutrition (body mass index of less than 20 kg/m^2 and/or serum total protein under 6.5 g/dL). Other exclusion criteria included high alcohol consumption (over 80 g/day) and treatment with drugs that could affect muscle structure or function (basically systemic steroids and beta agonists, high doses of inhaled corticosteroids, theophylline, calcium antagonists, and diuretics). The protocol, designed to comply with the Helsinki declaration on medical research involving human subjects, was approved by our hospital's Ethics and Clinical Research Committee. All participants signed the necessary informed consent form after receiving detailed information about the aims of the study and the possible risks involved in the techniques used.

Study Design

This was a cross-sectional study of muscle characteristics and function carried out over a period of 2 years.

On the first day, candidates for inclusion in both groups were questioned and examined. Nutritional and anthropometric data were studied before patients were admitted to the study. Subsequently, all candidates underwent conventional lung function testing, and respiratory muscle strength was also assessed.

Approximately 5 days later, a biopsy was performed on all the patients in both groups, either in the thoracic surgery operating theatre (for TLL) or in our laboratory (for AT).

The muscle samples were later processed simultaneously.

Procedures

1. Conventional lung function testing and assessment of respiratory muscle function.

Forced spirometry including a bronchodilator test was performed using a Datospir 92 spirometer (Sibel, Barcelona, Spain) to provide the data needed to determine static lung volumes and airway resistance after body plethysmography (Masterlab, Jaeger, Würzburg, Germany). Carbon monoxide transfer was measured with a device incorporated into the same apparatus. Arterial blood samples were taken for blood gas analysis (ABL 330 analyzer, Radiometer, Copenhagen, Denmark). The strength of each patients' respiratory muscles was assessed by measuring maximal inspiratory and expiratory mouth pressure using a manometer equipped with a mouthpiece that could be occluded (Sibelmed-163, Sibel, Barcelona, Spain). Both maximal pressures were measured during a static maneuver, maximal inspiratory pressure at residual volume and maximum expiratory pressure at total lung capacity. Published reference values valid for the Caucasian Mediterranean population were used for spirometry, plethysmography, carbon monoxide transfer, and respiratory pressure.[16-19]

2. Muscle Biopsy:

– TLL Method. Thoracotomy was performed under general anesthesia. After dissection of the superficial layers of the wall in the fifth intercostal space on the side of the lesion, a sample of the external intercostal muscle was taken using dissection scissors. The surgical procedure was then performed, involving opening the pleural cavity and consequent pulmonary collapse.

– AT Method. After infusing a local anesthesia (lidocaine 5%), samples were taken from the external intercostal muscle, also in the fifth intercostal space (anterior axillary line), using the technique described previously by our group.[14] The side to be sampled was selected randomly in each case. The technique used to obtain the sample started with a cutaneous incision and dissection by layers until the muscle was located. Then a few drops of lidocaine 2% were applied, after which the sample was taken with dissection scissors.

The biopsies measured approximately 5×5×5 mm and each one was immediately divided into thirds. The first part of each sample was transferred to a cryovial, frozen in liquid nitrogen, and stored at –80°C until it was used to analyze TNF-α and IGF-1 expression. The second part was embedded in optimal cutting temperature (OCT) cryocompound, frozen in isopentane cooled with liquid nitrogen, and later sectioned with a microtome (Cry-cut 2800N, Reichert-Jung, Nusslock, Germany) into 6-µm thick slices, which were then mounted on a slide using chrome alum gelatin and used in the enzyme histochemical analysis. The third piece was immersed in formaldehyde, embedded in paraffin, and sliced with a microtome into 3-µm sections, which were then mounted on a slide pretreated with 3-amino-propyltriethoxysilane and used in the immunohistochemical analysis.

Analysis of Fiber Size and Proportions

Fiber size and proportions were determined using immunohistochemical and enzyme histochemical analysis, followed by morphometric analysis. In order to confirm correct fiber orientation and assess the general characteristics of the muscle, the samples for the immunohistochemical analysis were stained with hematoxylin. Monoclonal antibodies specific for isoforms I and II of the myosin heavy chains (MyHC) (Biogenesis, New Fields, Poole, United Kingdom) were then used. Antigen-antibody binding was detected using the traditional avidin-biotin-peroxidase complex. The necessary negative controls were obtained by omitting the first antibody. Once the cell types had been labeled, morphometric analysis was performed using an optical microscope (Olympus, Series BX50F3, Olympus Optical Co., Hamburg, Germany), connected to an image digitalization system (Visual Communication Suite, Pixera Studio version 2.0, Pixera Corporation, Los Gatos, California, USA). The proportions of different cell types were determined, and fiber size was measured in at least 100 fibers in each sample using the public domain software developed by the US National Institutes of Health (NIH Image, version

1.60, Bethesda, Maryland, USA).

Samples for the enzyme histochemical analysis (adenosine triphosphatase [ATPase] assay) were incubated in a solution of 0.1 M sodium veronal, 0.10 M calcium chloride, and distilled water for 15 minutes at room temperature. The solution was adjusted to 3 different pH values (9.4, 4.6, and 4.2). The samples were then incubated for 45 minutes in a solution similar to the one described above, but with the addition of adenosine triphosphate disodium salt, also pH-adjusted. They were then rinsed successively in calcium chloride, copper chloride, sodium veronal, distilled water, ammonium sulphate, and, once again, in distilled water. The samples were then dehydrated with progressive concentrations of alcohol and finally washed in xylol.

Analysis of the TNF-α and IGF-1 Gene Expression in the Muscle

The technique used to analyze TNF-α and IGF-1 gene expression in the muscle was real-time polymerase chain reaction. Gene expression was determined by isolation of the RNA and reverse transcription followed by polymerase chain reaction.

RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY, USA). Briefly, 15 to 50 mg of tissue was homogenized in 1 mL of TRIzol reagent. Then 200 µL of chloroform was added, and the sample was shaken vigorously for 15 seconds and incubated at room temperature for 10 minutes. Samples were then centrifuged at 12 000 g for 15 minutes at 4ºC, after which 400 µL of the watery supernatant was transferred to a new tube. RNA was then precipitated with isopropanol, and the resulting sample was incubated at room temperature for 10 minutes and centrifuged again at 12 000 g and 4ºC. The resulting RNA pellet was washed with 70% ethanol, air dried, and resuspended in water free of ribonuclease. The concentration of RNA in the sample was determined by spectrophotometry, after which it was stored at –80ºC until the polymerase chain reaction was performed.

For the synthesis of complementary DNA (cDNA), 1 µg of total RNA was reverse transcribed in a 10 µL reaction using an oligo(dT)$^{12\text{-}18}$ primer and reverse transcriptase (Superscript II, Invitrogen, Life Technologies). After annealing for 10 minutes at 70ºC, reverse transcription was carried out at 42ºC for 50 minutes. The process was interrupted, and the temperature raised to 70ºC for 10 minutes. The whole process was performed with the GeneAmp PCR system (Perkin Elmer, Richmond, VA, USA).

Amplification was carried out on 1/50 aliquots of the cDNA produced in the previous phase using a sequence detector (ABI PRISM 7900HT, Applied Biosystems, Foster City, CA, USA). TNF-α and IGF-1 expressions were quantified with 2 preformulated assays (Taqman Assays-on-Demand Gene Expression Products, Applied Biosystems). The primers were selected on 2 different axons for each gene in order to avoid contamination with genomic DNA, and they were labeled with FAM (6-carboxyfluorescein) on the 5' end and with TAMRA (6-carboxy-tetramethyl-rhodamine) on the 3' end. The cDNA of the beta-actin was used as an internal control to normalize the differences in the quantity of total cDNA in each sample. The samples were arrayed in triplicate in a 384-well plate (Applied Biosystems). The reactions were performed with 1 µL of cDNA, 10 µL 2 × of the TaqMan master mix, 900 nM of each of the primers, and 250 nM of the fluorescent probe. The mixture was decontaminated by incubation at 50ºC for 2 minutes with AmpErase uracil N-glucosylase. The DNA-polymerase AmpliTAq Gold was then activated by raising the temperature to 95ºC for 10 minutes. Finally, 50 amplification cycles were carried out, each one comprising initial denaturation at 95ºC for 15 seconds and primer annealing-extension at 60ºC for 1 minute. The results were processed with a specific program (Sequence Detector Software 2.1, Applied Biosystems). Each gene under study was compared with the calibration DNA. The mean values of each gene under study were normalized with respect to the endogenous reference (beta-actin) in a threshold cycle (CT) to obtain the ΔCT value. The ΔCT of the calibration cDNA was subtracted from this to obtain the ΔΔCT value. The number of copies of each cDNA was calculated using the expression 2–(ΔΔCT). Resulting median values were used in each case although only results showing differences of less than 5% were used in calculations.

Statistical Analysis

The variables are shown as means (SD). The differences

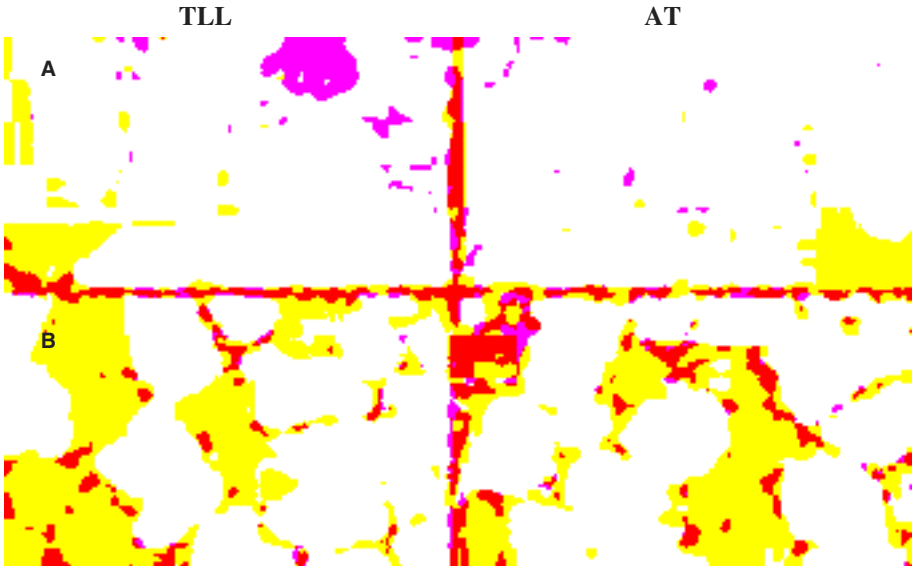

Figure 1. Cross sections of preparations from muscle samples obtained during thoracotomy performed because of a localized pulmonary lesion (TLL), and from samples obtained by ambulatory thoracoscopic (AT) biopsy. *a)* Immunohistochemical assay with identification of antibodies specific for myosin heavy chain isoform I (MyHC-1), and *b)* Adenosine triphosphatase (enzyme histochemistry).

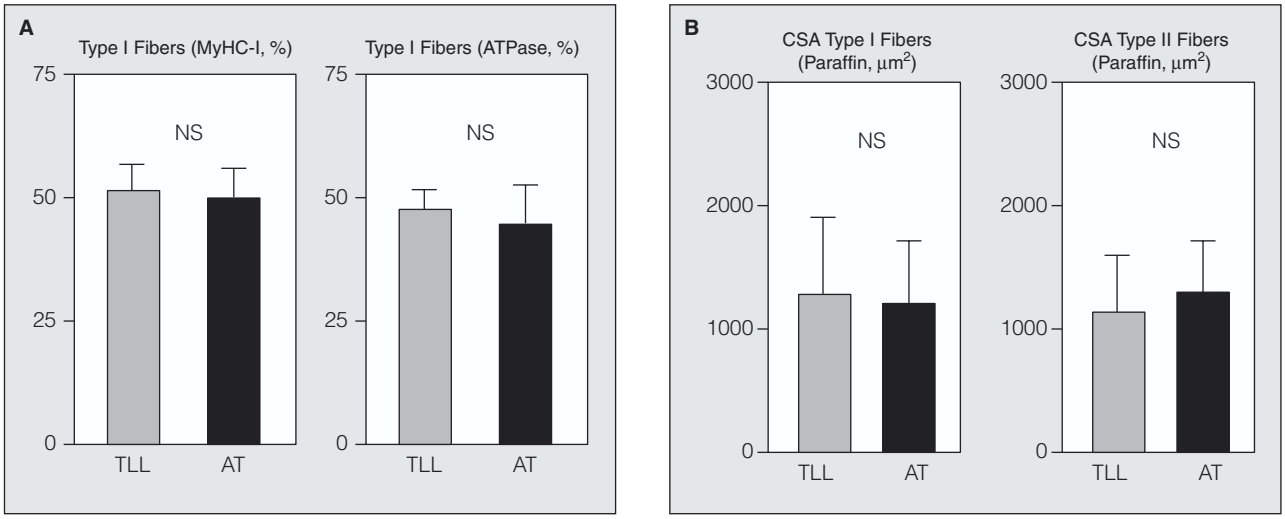

Figure 2. ***a)*** **Proportions of oxidative fibers obtained using immunohistochemical techniques and enzyme histochemistry in muscle samples obtained using the 2 methods under study (monoclonal antibodies specific for myosin-heavy-chain (myHC) isoform I or myHC and adenosine triphosphatase (ATPase); and** ***b)*** **fiber cell sizes in paraffin embedded samples. CSA indicates cross-sectional area; NS, not significant; TLL, thoracotomy performed because of localized lesion; and AT, ambulatory thoracoscopic biopsy.**

TABLE
Characteristics of the Patients Enrolled in the Study*

	TLL	AT
No. of patients	8	7
Age, years	68 (5)	64 (6)
Weight, kg	74 (11)	79 (7)
BMI, kg/m^2	25 (3)	27 (3)
FEV_1, mL	1575 (416)	1671 (375)
FEV_1, % predicted	47 (9)	50 (10)
FEV_1/FVC, %	61 (13)	66 (9)
RV/TLC, %	5 (7)	55 (6)
DLCO, % predicted	74 (15)	70 (19)
PImax, % predicted	62 (12)	65 (14)
PEmax, % predicted	74 (13)	71 (16)
PAO_2	73 (7)	77 (11)
$PaCO_2$	37.2 (4.4)	39.2 (1.19)

*Values are shown as means (SD). No significant differences were detected between the 2 groups. TLL indicates thoracotomy performed because of localized lesion; AT, ambulatory thoracoscopic biopsy; BMI, body mass index; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; DLCO, diffusing capacity of carbon monoxide; PImax, maximal inspiratory pressure; PEmax, maximal expiratory mouth pressure; PAO_2, partial alveolar pressure of oxygen.

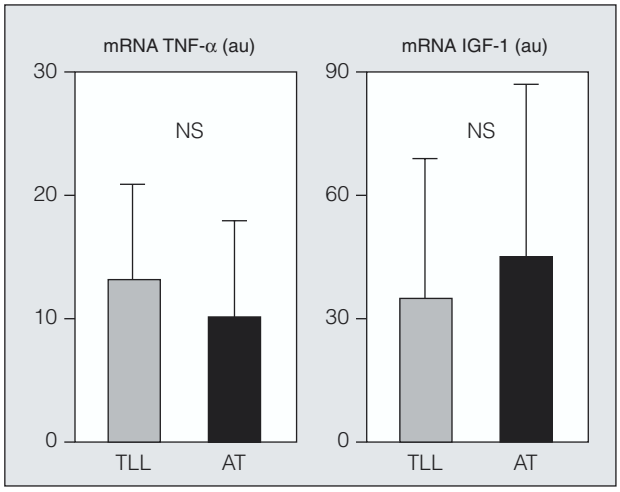

Figure 3. Values relating to the expression of the tumor necrosis factor alpha genes (TNF-α) and insulin-like growth factor (IGF-1) in external intercostal muscle samples obtained by thoracotomy performed because of a localized lesion (TLL) and by ambulatory thoracoscopic (AT) biopsy in patients with chronic obstructive pulmonary disease. mRNA indicates messenger RNA; au, arbitrary units; NS, not significant.

between the 2 groups were analyzed using the nonparametric Mann-Whitney U test, and possible correlations between different variables were evaluated using Spearman' correlation coefficient. The significance level was $P<.05$ in all cases.

Results

The general characteristics of the patients in both groups are shown in the Table. Basically, the participants were individuals whose nutritional status was normal but who had moderate to severe lung impairment characterized by severe airway obstruction, pulmonary hyperinflation, moderate gas exchange impairment, and weaker than normal respiratory muscles.

The fibrous characteristics of the external intercostal muscle were similar in both groups of patients (Figures 1 and 2). A slight predominance of oxidative fibers was found with both the classification methods used (immunohistochemical assay and ATPase). No differences in size were observed between the intercostal samples taken during TLL and those obtained by AT. In both cases, the cross-sectional areas found could be considered normal. It should be noted, however, that cross-sectional areas were measured using the paraffin-embedded samples, and they are therefore somewhat smaller than those that would be obtained using the samples that were frozen and

embedded in OCT owing to a number of different factors inherent in each technique. In particular, the former involves a certain degree of dehydration and the latter is characterized by the frequent presence of artifacts of freezing. In general, if correct procedure is used in both cases, the latter has a correction coefficient that varies from 1.3 to 1.6 in our laboratory.

The expression of the TNF-α and IGF-1 genes was also similar in both groups of patients (Figure 3), and no correlations of interest were found with respect to fiber structure.

Given the relative functional and nutritional homogeneity in the population studied, no significant correlations of interest were found between this type of variable and the structural or expression characteristics of the muscle.

Discussion

This study supports the use of the TLL method for obtaining and evaluating samples of respiratory muscle in patients with COPD. In addition to confirming the expected result—that in carefully selected patients the fiber phenotype is not affected by the underlying disease—we also found no detectable effect on the local expression of TNF-α or IGF-1. These findings lead us to conclude that samples obtained during TLL are as useful as those obtained by AT for the study of processes such as the local expression in respiratory muscles of cytokines and growth factors. Furthermore, the absence of any differences between the intercostal samples obtained using these 2 methods allows us to postulate that prior studies carried out using diaphragm samples obtained during TLL are probably valid.

It is known that the respiratory muscles of patients with COPD are capable of developing even greater strength than those of healthy individuals with lung capacities similar to those of COPD patients.[6] This led us to consider the possible existence of structural and metabolic adaptations in the respiratory muscles of COPD patients. Such adaptations might to some degree counteract the harmful effects of the increase in loads, changes in muscle geometry (away from the optimum contraction length), and the resulting imbalance between the supply and demand of nutrients to the muscle.

However, obtaining respiratory muscle samples suitable for structural and protein expression studies is fraught with a series of problems associated with the sampling method used. Originally, as mentioned in the introduction, samples were taken from cadavers.[9,20] This method has been progressively abandoned because, although it may be useful for the study of very static properties (eg, fiber proportions), it is not appropriate for the study of the many processes susceptible to change in the presence of factors that may accompany biological death. Neither is it possible in most cases to determine with sufficient certainty whether or not the patient was suffering from COPD, or to ascertain the individual's degree of functional impairment prior to death. Furthermore, it is not always possible to rule out or obtain sufficient information concerning possible comorbidity. As an alternative to autopsy, samples can be obtained during thoracotomy performed to remove a localized pulmonary lesion. This is the method that has been most often used[5,11,12,21-23] and it has undeniable advantages. Firstly, it allows scientists to obtain samples of any respiratory muscle they need to study. Secondly, a precise diagnosis of the lung disease is available, including the function and state of the parenchyma. Moreover, using TLL samples can be obtained of respiratory muscles from patients with normal lung function. There are, nonetheless, some limitations. The most important of these is the inevitable presence of comorbidity in the form of the disease that gave rise to the need for surgery, almost always a neoplastic process. Although malnutrition and paraneoplastic disorders can be ruled out to a reasonable degree by careful selection of patients, they cannot be totally excluded. Moreover, many likely candidates for TLL whose lung function is still within the broad limits we consider normal have been smokers—hence their lung cancer—so that they can only be seen as "controls" purely from the standpoint of lung mechanics. It is possible that their muscles have already started developing the deficiencies associated with tobacco use and/or the initial stages of COPD even though their lung function variables may still be substantially normal.

The next sampling method proposed was muscle biopsy during lung-volume-reduction surgery.[4] This method is useful in many cases because it allows the researcher to exclude serious comorbidity. Moreover, it can facilitate the longitudinal study of the effects of the surgery on the thoracic muscle structure if a second biopsy is taken some time after surgery has been performed. However, there are 3 fundamental problems associated with this method. Firstly, samples cannot be obtained from subjects with normal lung function or with only slight to moderate impairment. This rules out the possibility of having a control group and studying the progression of muscular involvement in the various stages of the disease. Furthermore, in most hospitals patients undergo a rehabilitation program of respiratory physical therapy prior to surgery, which probably distorts many of the findings related to muscle conditioning or deconditioning. It has been observed that respiratory rehabilitation, and specifically respiratory muscle training, brings about changes in muscle fiber properties and in the expression of inflammatory cytokines.[24] Furthermore, after a systematic review of the results of lung reduction surgery,[10] most programs have been drastically reduced, making it difficult to obtain samples and at the same time posing serious ethical questions with respect to this procedure.

The idea has also been put forward, although only on a theoretical level, of obtaining samples from patients with advanced COPD in the course of lung transplants. It is, however, not difficult to rule out this method because of the important bias that would be introduced

by the indispensable immunodepressant therapy.

Some years ago, the limitations described above led us to develop a new method of obtaining samples by way of upper laparotomy.[13] After developing a surgical technique that would eliminate the principal technical problem[25] —the provocation of a iatrogenic pneumothorax—this method looked promising because it made it possible to rule out the presence of serious comorbidity. However, in practice it has proven less useful than expected because the indications for laparotomies have been drastically reduced due to the increased use of laparoscopic surgery, a less costly technique associated with lower risk. In effect, laparotomy is now hardly ever used outside the context of emergency operations and tumor removals. It is difficult, however, to obtain samples during emergencies because of the frequent presence in such cases of hemorrhage or perforation, and even when samples can be obtained their validity remains doubtful. In the case of surgery performed to remove a tumor, the problem of serious comorbidity comes up again because of the underlying disease.

In this context, our group once again developed a method for obtaining samples from patients who could be preselected in accordance with an ideal study design.[14-26] This method—AT biopsy—is minimally invasive, is performed on outpatients, and allows us to obtain samples of different respiratory muscles, such as the intercostal and the external oblique muscles. Furthermore, the outcome of an intervention can be studied by repeated sampling of the same subject[24] since it has been demonstrated that the phenotypes of the dominant and nondominant sides of the thorax are identical at this level.[14] There is, however, one serious problem associated with this method: it cannot be used to obtain samples of the diaphragm, which is the principal respiratory muscle.

In light of this analysis, it seems clear that the most appropriate method for sampling any respiratory muscle, including the diaphragm, is TLL. The question that remains, however, is whether, after careful selection of patients in order to rule out confounding factors, this method is, in fact, valid for studies undertaken to analyze the changes that occur in these muscles in the course of COPD. This question is of particular interest now that the phenotypical characteristics of the respiratory muscles in this disease appear to have been well defined, and the current objectives of research are more focused on the study of other properties, such as the presence of oxidative stress,[7,27,28] the local expression of cytokines,[8] apoptosis,[29] and the expression of growth factors.[30] One possible way to approach this question is to analyze some of these factors in samples of muscles that can be biopsied using both the TLL and the AT methods (the latter being a model that makes it possible to completely eliminate comorbidity). A case in point is the external intercostal muscle, which, although not the principal respiratory muscle, plays an important role in breathing, particularly when the loads or demands of the system are high.[31-33] If a difference were observed between the fiber phenotypes of samples obtained during TLL and those obtained by way of AT, this would raise a doubt concerning the results reported in the literature and generally accepted as valid. If the discrepancy affects the expression of cytokines or growth factors, new methods for obtaining samples should be developed if researchers wish to investigate these factors in COPD, or else animal models should be used to draw conclusions about this disease. Conversely, if the results obtained using both methods were similar, this would confirm the findings concerning the fiber phenotype of respiratory muscles obtained using TLL samples, and furthermore future studies of other phenomena, such as those mentioned above, can be envisaged. Moreover, the use in such studies of diaphragm samples obtained during TLL would be supported albeit indirectly (since it is not the muscle analyzed in the present study).

On the basis of our results, which were very similar for both TLL and AT samples, we can confirm that the results of structural studies of muscle samples obtained during TLL are valid. Moreover, although more substances should be analyzed in future studies, the analysis of cytokine and growth factor expression using such samples are also valid. As was hypothesized above, this statement probably holds true not only for the external intercostal muscle but also for the diaphragm, since our results appear to demonstrate the insignificance of the influence exercised by the underlying disease on muscle characteristics.

The structural properties of the fibers are the main factors influencing the structural properties of the muscle. Anaerobic fast-twitch fibers—type II fibers that express predominantly MyHC-II—are capable of producing strong contractions for a limited period of time.[34] Conversely, slow-twitch aerobic fibers—type I fibers with a predominance of MyHC-I—are more resistant to fatigue but have less contractile strength.[34] Fiber size also directly influences contraction strength. Speaking generally, we could say that a muscle with larger fibers (in particular if they are type II) and with a larger proportion of type II fibers will develop more contractile strength.[34]

TNF-α is a proinflammatory cytokine with multiple actions. On the one hand, it actively promotes the immune response and antitumor activity and, under certain conditions, it can induce angiogenesis and contribute to cell proliferation and migration.[35] On the other hand, an increase in TNF-α can have harmful effects; it can reduce structural protein synthesis (eg, myHC), accelerate catabolism, induce apoptosis, and directly inhibit muscle contractility.[36] In addition, TNF-α is closely linked to the presence of oxidative and nitrosative stress, both present in the muscles of patients with COPD.[7] In fact this cytokine can act as a systemic factor (endocrine effect), synthesized by various cells of the immune system or by body fat, and also as a factor

of local action (paracrine and autocrine effects) since it is synthesized by the muscle itself.[37] TNF-α has been implicated in the loss of muscle mass and function that accompanies various processes including aging, sepsis, COPD, and cancer. A fundamental factor in its expression in muscle is the level of preceding activity. We recently demonstrated that aging, which is linked to a sustained increase in activity owing to changes in the mechanical properties of the respiratory system, produces an underexpression of this factor in the ventilatory muscles of older people.[38] Vigorous exercise, on the other hand, produces local overexpression of TNF-α even in healthy individuals.[39] The topic is still under discussion since other authors have not observed the described increase in either healthy individuals or COPD patients after moderate exercise, at least in leg muscles.[8] These discrepancies are probably due to the prior condition of the muscle studied (trained as opposed to unfit) or to the intensity of the exercise.

IGF-1 is probably the principal muscle growth factor, and it appears to be involved in both repair and growth.[40] Like TNF-α, its actions can be endocrine (fundamentally those of the IGF-1 synthesized in the liver and distributed throughout the whole organism) or autocrine-paracrine, (actions exercised by the IGF-1 synthesized locally in the muscle itself).[40] Of the latter, there exist 2 isoforms that are very similar structurally but that are regulated differently: classic IGF-1 (called IGF-IEa), with actions similar to the systemic IGFs, and the so-called mechano growth factor (MGF or IGF-IEc).[41] MGF seems to play a key role in the activation of genes linked to structural proteins in response to mechanical stimulus, while the classic form responds more to chemical signals. There is also cross-talk between IGF-1 and TNF-α: IGF-1 appears to inhibit the activation of nuclear factor-kappaB that is induced by TNF-α.[42]

Based on the results obtained in the present study of the external intercostal muscle for all 3 variables (fiber structure, TNF-α expression, and IGF-1 expression), the TLL and AT sampling methods have been shown to be interchangeable.

The present study is limited in 2 ways. Firstly, only a small number of substances were analyzed. Although the potential number of substances susceptible to analysis is almost infinite, the team decided to focus on only 3: basic fiber structure and the expression of a representative of each of 2 families; a cytokine and a growth factor. In the case of fiber structure, the aim was to establish the validity of most of the data published in the literature to date; and in the case of the cytokine and the growth factor the aim was to gain some understanding of the situation with respect to these types of substances, which are of great interest in current research. Nonetheless, in spite of our results, it would seem advisable to carry out further studies analyzing a wider range of substances. The second limitation is that it was not possible to study samples of the main respiratory muscle—the diaphragm—obtained by TLL in comparison with samples of this muscle obtained using another method which would allow the exclusion of comorbidity. However, this study design is rendered impossible by the characteristics and limitations of all of the sampling methods currently in use. On the other hand, the results we obtained for the intercostal muscle allow us to infer that the situation is probably similar in the diaphragm, since any systemic effects of the underlying disease should have the same influence on both muscles.

The conclusion arising from the present study is that we can confirm the validity of the TLL method of obtaining respiratory muscle samples for both structural studies and, probably, for the analysis of inflammatory cytokine and growth factor expression.

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