Developmental myosin heavy chains in the adult human diaphragm: coexpression patterns and effect of COPD

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Developmental myosin heavy chains in the adult human diaphragm: coexpression patterns and effect of COPD. J. Appl. Physiol. 88: 1446–1456, 2000.—In preliminary experiments we noted developmental (i.e., embryonic and neonatal) myosin heavy chains (MHCs) in the diaphragms of patients with severe chronic obstructive pulmonary disease (COPD). We hypothesized that this finding represented new fiber formation secondary to injury associated with the mechanical stress of COPD or previously undescribed MHCs in the human diaphragm. To distinguish between these possibilities, we analyzed diaphragmatic biopsies obtained from 9 patients with severe COPD (forced expiratory volume in 1 s = 21 ± 2% predicted, residual volume = 283 ± 22% predicted) and 10 age-matched controls. First, using immunocytochemistry with specific monoclonal antibodies, we noted that control diaphragms had greater proportions of fibers expressing embryonic (50 ± 2 vs. 28 ± 3%, P < 0.0001) and neonatal (52 ± 2 vs. 32 ± 3%, P < 0.001) MHCs than COPD diaphragms. Second, SDS-PAGE demonstrated that these developmental MHCs represented only a very small fraction of the diaphragmatic MHC content. Third, the RT-PCR demonstrated mRNA coding for embryonic and neonatal MHCs in COPD and control diaphragms. Last, COPD and control diaphragms exhibited normal histology on light microscopy. We conclude that the presence of developmental MHC isoforms does not indicate new fiber formation in diaphragms of patients with severe COPD. Although these results represent the first systematic description of embryonic and neonatal MHCs in normal adult human diaphragms, their function remains to be elucidated.

human diaphragmatic muscle; embryonic myosin heavy chain; neonatal myosin heavy chain; fiber types; immunocytochemistry

IN 1977, Roussos and Macklem (39) demonstrated that the diaphragm, the major inspiratory muscle of humans, can develop fatigue. Although the site of muscular fatigue can occur anywhere in the motor pathway between the cerebral cortex and the muscle, previous workers have demonstrated that a particular type of fatigue, i.e., low-frequency fatigue, occurs at the level of the muscle cell (6, 18). Bellemare and Bigland-Ritchie (3) and others (2, 13, 23, 28, 32) demonstrated that various types of exercise can elicit low-frequency diaphragmatic fatigue in normal subjects. Surprisingly, the literature does not contain any references to exercise-induced, low-frequency diaphragmatic fatigue in patients with chronic obstructive pulmonary disease (COPD). Indeed, the study of Polkey et al. (35) showed that patients with severe COPD [i.e., those with forced expiratory volume in 1 s (FEV1.0) < 50% predicted (1)] did not develop low-frequency diaphragmatic fatigue during treadmill exercise to “exhaustion.”

De Troyer et al. (5) demonstrated that neural drive to the diaphragm is increased in patients with severe COPD. Moreover, Bellemare and Grassino (4) demonstrated that the diaphragms of patients with severe COPD have a higher energy expenditure, as evaluated by the diaphragmatic time-tension index, than the diaphragms of normal subjects. In 1997, we hypothesized that this combination of a chronically increased energy expenditure and relative resistance to low-frequency fatigue (in diaphragms of patients with severe COPD) might be accounted for by COPD-induced cellular adaptations characterized by an increase in the proportion of slow-twitch diaphragmatic fibers (i.e., type I), which are fatigue resistant, and by decreases in the proportion of fast-twitch diaphragmatic fibers (i.e., types IIa and IIb), which are fatigable. Recently, we provided evidence that the diaphragms of patients with severe COPD do exhibit these hypothesized adaptations (24). However, our previous work does not elucidate the mechanism underlying these COPD-induced adaptations of the human diaphragm.

Reid et al. (38) used a model of increased airway resistance elicited by tracheal banding to demonstrate diaphragmatic injury. Keens et al. (17) and Prezant et al. (36) showed that tracheal banding elicits a transformation from fast- to slow-twitch fiber type. The combination of these results suggests that diaphragmatic injury can elicit transformations from fast- to slow-twitch fiber type. One pathway for this to occur involves the transformation of satellite cells into myotubes expressing developmental myosin heavy chains (MHCs),

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which then differentiate into mature slow-twitch fibers (43, 44). In preliminary experiments, we noted the presence of developmental MHCs in the diaphragms of patients with severe COPD. These observations raised the possibility that these developmental MHCs (i.e., embryonic and neonatal) were due to new fiber formation secondary to injury associated with the mechanical stress of COPD. Alternatively, these developmental MHCs may indicate the presence of previously undescribed MHCs in the adult human diaphragm. To distinguish between these hypotheses, we carried out the present study to characterize the diaphragms of patients with severe COPD, as well as those of age-matched controls, with respect to the expression of these developmental MHCs.

METHODS

Subjects

Our COPD group consisted of five men and four women with severe COPD who were undergoing lung volume reduction surgery. In contrast, our control group was composed of three men and seven women with mild pulmonary impairment [i.e., FEV₁ 1.0 = 70–100% predicted (1)] who were undergoing resection of solitary pulmonary nodules.

Pulmonary Function Tests

Before surgery, all subjects underwent pulmonary function testing. Spirometry and plethysmographic lung volumes were measured by conventional techniques, and values were compared with predictions (16, 19).

Diaphragm Biopsies

A full-thickness biopsy was obtained from the right anterior costal diaphragm region lateral to the insertion of the phrenic nerve. The biopsy specimen was ~20 mm long and 10 mm wide; the length was oriented in the direction of the fibers radiating from the central tendon. Each biopsy was cut lengthwise into halves: one half was used for immunohistochemistry, and the other was allocated for biochemical measurements. The halves were then laid on a small thin sheet of aluminum foil, gently flattened with forceps, and immersed (for 8–10 s with gentle agitation) into 250 ml of isopentane that had been cooled by liquid nitrogen to a slushy appearance. The time between the excision of the samples and the immersion in isopentane ranged from 3 to 5 min. The frozen samples were then transferred to liquid nitrogen to complete the freezing process. All diaphragm samples were wrapped in aluminum foil and stored at −70°C until analyzed.

Informed consent for these biopsies was obtained from each of the subjects, and our protocol was approved by the Institutional Review Boards of the Philadelphia Veterans Affairs Medical Center and the Hospital of the University of Pennsylvania.

Analytic Techniques

We used the following three types of analyses: 1) immunocytochemical measurements of the proportions of fibers expressing different MHCs and categories of MHC-determined fiber types, 2) SDS-PAGE and Western blots for developmental MHCs, and 3) RT-PCR assays of mRNA coding for the different MHCs.

Immunocytochemistry

Preparation of tissues. Briefly, we prepared serial frozen 10-μm sections with a cryostat (HistoSTAT Microtome-855, AO Scientific Instruments, Buffalo, NY) maintained at −22°C; sections were then kept at −70°C until they were stained.

MHC-determined fibers. Our staining protocols have been previously described (24, 33). Briefly, fiber types were classified by indirect immunofluorescence with monoclonal antibodies specific for the following MHCs: N007.5.4D for slow-twitch (i.e., type I) fibers (33), SC-71 for type IIa fibers (45), BF-F3 for type IIb fibers (45), F4–286 for embryonic MHC (10), and BF-34 for neonatal myosin (S. Schiaffino, personal communication).

Using the above-noted procedures, we were unable to unequivocally determine the presence or absence of MHC IIb (i.e., BF-F3 reactivity) in all fibers. Accordingly, for this determination, we used a signal amplification system (Renaissance TSA-Indirect, NEN Life Science Products, Boston, MA).

For the remaining analyses, we used a signal amplification system (Renaissance TSA-Indirect, NEN Life Science Products, Boston, MA).

Image analysis. Our system consisted of the following components: 1) a light microscope (Diaplan, Leitz, Wetzlar, Germany) fitted with a 100-W fluorescent light source, 2) a digital color charge coupling device camera with a pixel resolution of 8.3 × 8.3 μm (model DC-100, Leica, Deerfield, IL), 3) a 300-MHz desktop computer (E-3100 series, Gateway-2000, Sioux Falls, SD) equipped with a 24-bit RGB PCI frame grabber board (Leica), and 4) a high-resolution [i.e., 1,640 (horizontal) × 1,280 (vertical) lines] 21-in. color monitor (Vivitron, Gateway-2000).

Preliminary observations. In our initial studies, we noted that multiple MHCs were expressed in the same fiber on serial cross sections. These observations could be due to longitudinal variation of MHC expression along the length of the fibers or expression of multiple MHCs in the same sarcomere. Because this point was of critical importance to our study protocol, we studied immunocytochemically stained longitudinal sections of COPD and control diaphragms, and we noted no longitudinal differences in MHCs in control or COPD diaphragms. Therefore, our use of a cross-sectional analysis should not affect our statements regarding MHC coexpression. Accordingly, all immunocytochemical results in this study were obtained from serial cross sections.

Quantitation of fiber-type proportions. We digitized all portions of each serial section, and these digital images were arranged to create complete representations of each of the serial sections. In this manner, we were able to trace >95% of the individual fibers through all serial sections. At least 400 fibers were traced through each series of cross sections; however, variation in fiber type proportions was negligible after ~200 fibers were counted.

SDS-PAGE and Immunoblotting

MHC proportions. Myosin for electrophoresis was prepared by mincing the muscle tissue with scissors in a high-salt solution with a pH of 6.5 at 4°C and incubating the tissue for 40 min. Extracts were centrifuged, and supernatants were recovered for electrophoresis. Electrophoresis of MHC isoforms was performed using a specific modification of previously described protocols (20, 21, 27).
Electrophoresis was subsequently performed on slabs (18 cm × 16 cm × 0.75 mm thick) with use of previously described stacking and separating gels (27). Lanes were loaded with 1–3 µl of myosin extract that contained 0.5–1.5 µg of protein. Electrophoresis was carried out for 22–24 h at 120 V with a vertical slab unit (model SE600, Amersham Pharmacia Biotech, San Francisco, CA) with Tris-glycine running buffer (pH 8.3) maintained at 15°C (Endocal RBC-3, Neslab, Portsmouth, NH). After completion of electrophoresis, gels were silver stained.

The blotting procedure was a modification of that described by Towbin et al. (51). Briefly, MHC bands from 5% polyacrylamide gels were electrophoretically transferred to a nitrocellulose sheet. The filters were incubated at room temperature with the above-noted antibodies for embryonic and neonatal MHCs for 2 h and visualized with a blotting detection kit (RPN.22, Amersham Pharmacia Biotech), which is based on biotinylated secondary antibody (anti-mouse) detected with a streptavidin-alkaline phosphatase conjugate.

PCR Analysis

RNA preparation and cDNA synthesis via RT. Total RNA was isolated from pulverized frozen diaphragm samples by acid guanidinium thiocyanate-phenol-chloroform extraction, commercially available in a kit (TRIzol Reagent, Life Technologies, Gaithersburg, MD). RNA integrity was ensured by noting the characteristic rRNA bands on 1% denatured agarose gel electrophoresis. Three micrograms of total RNA were reverse transcribed in a 20-µl reaction mixture containing 1× RT buffer (50 mM Tris·HCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3), 10 mM dithiothreitol (DTT), 0.5 mM dNTPs, 0.5 µg of oligo(dt) (Promega, Madison, WI), 40 U of RNase inhibitor (Recombinant RNasin RNase Inhibitor, Promega), and 200 U of Superscript II RT (Life Technologies). The reaction was carried out at room temperature for 10 min, then incubated at 42°C for 60 min. At the end of the reaction, the mixture was heated to 75°C for 15 min to deactivate the RT.

PCR. Each PCR amplification was carried out in 50 µl of total volume containing 2 µl of RT products as template, 1× PCR buffer (10 mM Tris·HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTP, 2 U of AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA), and 8 µl of each of the appropriate 5’- and 3’- oligonucleotide primers. Table 1 shows the base pairs for the 5’- and 3’-primers used in the PCR reactions. PCR amplifications were performed for 30 cycles with an automated GeneAmp PCR System 9600 (PE Applied Biosystems). Each cycle consisted of three phases: 30 s at 94°C, 30 s at the appropriate annealing temperature for the primers used (Table 1), and 30 s at 72°C. After the 30 cycles, the PCR product mixture was maintained at 72°C for 5 min. Subsequently, these PCR products were subjected to electrophoresis on a 1% nondenaturing agarose gel, and then photographed under ultraviolet light.

Table 1. Characteristics of PCR primers used in identification of MHCs

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>MHC mRNA Amplified</th>
<th>Annealing Temperature, °C</th>
<th>Size of Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’-Primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNTCAAGAGAAAANTGC</td>
<td>I</td>
<td>50</td>
<td>209</td>
</tr>
<tr>
<td>TTCAAGAGACNNTNNGC</td>
<td>Iia</td>
<td>53</td>
<td>170</td>
</tr>
<tr>
<td>NCAGNTAAATATTTATTC</td>
<td>Iix</td>
<td>53</td>
<td>155</td>
</tr>
<tr>
<td>TGCAAAAATNTCCTGAG</td>
<td>Iib</td>
<td>52</td>
<td>155</td>
</tr>
<tr>
<td>AANNTTATTTGCAATGG</td>
<td>Embryonic</td>
<td>48</td>
<td>231</td>
</tr>
<tr>
<td>GTCAATAAGCAAAGTGACC</td>
<td>Neonatal</td>
<td>56</td>
<td>162</td>
</tr>
<tr>
<td>5’-Primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGCGAGGTCGGGANGAG</td>
<td>I, embryonic</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>AAGGTTCAACATAAAA</td>
<td>I, Ilx, Iib, neonatal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A, adenine; G, guanine; C, cytosine; T, thymine; N, randomly assigned nucleotide; MHC, myosin heavy chain. Annealing temperature was chosen to optimize product formation with indicated 3’-primers and their corresponding 5’-primers.

Statistical Analysis

Values are means ± SE. A multivariate ANOVA (MANOVA) was used to compute the statistical significance of differences between control and COPD diaphragms with respect to any set of measurements (31). If the MANOVA indicated statistically significant differences, then group t-tests were used to determine differences between control and COPD groups with respect to individual measurements. Differences not significant at the 0.05 level were attributed to chance.

RESULTS

Vital Statistics and Pulmonary Function Measurements

The COPD patients did not differ significantly from the control subjects with respect to age, height, or weight (Table 2). The COPD patients had greater residual volume, functional residual capacity, and total lung capacity than the control subjects, whereas the control subjects had higher FEV₁.₀, forced vital capacity, and ratio of FEV₁.₀ to forced vital capacity.
Immunocytochemical Measurements of MHC Expression

Comparison of representative COPD and control diaphragms with respect to proportion of fibers expressing different MHCs. Figure 1 compares serial sections from a representative control diaphragm and a representative COPD diaphragm with respect to the proportion of fibers expressing the different MHCs. In Fig. 1, A–H, the green fibers are those that react with the antibody specific for the MHC of interest, whereas the dark fibers are those that do not react with this antibody. A comparison of Fig. 1, A and B, shows that the COPD diaphragm contains a greater percentage of fibers reacting with the antibody specific for slow MHC (i.e., MHC I) than the control diaphragm. Comparison of Fig. 1, C and D, indicates that the control diaphragm contains a greater proportion of fibers reacting with the antibody specific for MHC IIa. Figure 1, E–H, indicates that COPD and control diaphragms contain large numbers of fibers reacting with the antiembryonic and antineonatal MHC antibodies; surprisingly, the control diaphragm contains greater proportions of these fibers.

Fig. 1. Staining of serial diaphragmatic cross sections for slow (type I), type IIa, embryonic, neonatal, and type IIb myosin heavy chains (MHCs). Left: results obtained from a diaphragm of a control subject. Right: results obtained from a diaphragm of a patient with chronic obstructive pulmonary disease (COPD). Cross sections were preincubated with the following antibodies: NOQ7.5.4D, specific for slow MHC (A and B); SC-71, specific for MHC IIa (C and D); F4–2B6, specific for embryonic MHC (E and F); BF-34, specific for neonatal MHC (G and H); and BF-F3, specific for MHC IIb (I and J). In A–H, green fibers are those that react with antibody specific for MHC of interest, whereas dark fibers are those that do not react with this antibody. Because of a different methodology (see METHODS), darker fibers in I and J are those that react with BF-F3 antibody, whereas light fibers are those that do not react with this antibody. X, fiber that reacts with only anti-MHC I antibody; Y, fiber that reacts with antibodies against type IIa, embryonic, neonatal, and type IIb MHCs. Scale bars, 50 µm.
than the COPD diaphragm. Fourth, because a different staining technique was used for the BF-F3 antibody (antibody specific for MHC IIb), the darker fibers in Fig. 1, I and J, indicate a positive reaction with this antibody, whereas the light fibers are those that do not react with this antibody. Comparison of Fig. 1, I and J, indicates that the control diaphragm contains a larger proportion of fibers reacting with the BF-F3 antibody than the COPD diaphragm. Additionally, Fig. 1, I and J, shows that BF-F3 reactivity is present only in those fibers reacting with the antibody specific for MHC IIa. Although this latter finding was true for all COPD and control diaphragms, we also noted some fibers that reacted with the antibody specific for MHC IIa but did not react with the BF-F3 antibody (not shown).

Qualitative comparison of representative COPD and control diaphragms with respect to coexpression patterns. Because serial sections were used for the data in Fig. 1, Fig. 1 provides information on coexpression patterns. The symbol X indicates a fiber that reacts only with the antibody for slow MHC, whereas symbol Y shows a fiber that reacts with antibodies against MHCs IIa, embryonic, neonatal, and IIb. Examination of the panels of Fig. 1 indicates that these two fiber types are the most common MHC-determined fiber types.

Quantitative comparisons of COPD and control diaphragms with respect to proportions of fibers expressing different MHCs. Figure 2 indicates that COPD diaphragms contained a greater proportion of fibers reacting with the antibody specific for slow MHC, whereas control diaphragms contained a greater proportion of fibers reacting with type IIa, embryonic, neonatal, and type IIb MHCs. A MANOVA indicated that the fiber-type proportions in COPD and control diaphragms were statistically different, and the nominal P values provide some measure of the statistical significance of differences between COPD and control diaphragms with respect to specific MHC-determined fiber types.

SDS-Polyacrylamide Gels and Immunoblotting

Figure 4, top, shows the 200-kDa range of an SDS gel. We (24) as well as others (27, 30) have used immunoblotting to demonstrate that in the control diaphragm the band with the greatest electrophoretic mobility (i.e., the bottom band) is composed of MHC I, the band with intermediate mobility is MHC IIa, embryonic, and neonatal MHCs in control diaphragms. A MANOVA indicated that the proportions of MHC-determined fiber types in COPD and control diaphragms were statistically different, and the nominal P values provide some measure of the statistical significance of differences between COPD and control diaphragms with respect to specific MHC-determined fiber types.

Fig. 2. Proportion of fibers in diaphragms containing slow, type IIa, embryonic (Emb), neonatal (Neo), and type IIb MHCs. Expression of these MHCs was determined by reaction of diaphragm fibers with isoform-specific antibodies (see Fig. 1). Numbers in ○ indicate specific control subjects; numbers in ● represent patients with COPD. Statistical comparisons were carried out between control and COPD diaphragm with respect to percentage of fibers expressing each of MHC isoforms: *P < 0.05; ***P < 0.001; ****P < 0.0001.
MHC IIb/x (see Discussion for rationale underlying labeling of this band).

Figure 4, top, shows that the MHC IIb/x band is absent in the COPD diaphragms but clearly visible in the control diaphragms. More importantly, the proportion of the MHC content accounted for by the MHC I band is greater in COPD than in control diaphragms (63 vs. 48%). These percentages are virtually identical to those noted in our previous study (24), and they are also consistent with the observations of Mercadier et al. (30).

To demonstrate the electrophoretic mobility of embryonic and neonatal MHCs on SDS gels, we used human fetal diaphragms: lane 6 shows results obtained with an 18-wk-old fetal diaphragm; lanes 7 and 8 show results from 28-wk-old fetal diaphragms. Lloyd et al. (27) demonstrated that in the 18-wk-old fetal diaphragm the bottom band (which is of lesser intensity) is composed of slow MHC, whereas the band with less electrophoretic mobility (i.e., the top band) is composed exclusively of embryonic and neonatal MHC. In contrast, Lloyd et al. also demonstrated that in the 28-wk-old fetal diaphragm the top band is composed of two subcomponents (i.e., an embryonic/neonatal and a type IIa MHC subcomponent). However, the top band in lanes 7 and 8 of Fig. 4 does not resolve these two components; rather, it shows a single band. Figure 4, bottom, shows the results of immunoblotting representative of control, COPD, and fetal diaphragms; it shows that our monoclonal antibody that is specific for embryonic MHC reacts only with the top band of the three fetal diaphragms. We interpreted the lack of reactivity of the control and COPD diaphragms as indicating that, under the experimental conditions of our SDS-PAGE procedure, our lanes did not contain enough embryonic MHC to give a positive reaction.

To test this conclusion, we sequentially increased the protein loaded per lane of SDS gels used for immunoblotting. When we reached protein concentrations of 15–20 µg/lane, we were able to demonstrate positive immunoblots for both developmental isoforms (i.e., embryonic and neonatal MHCs). Figure 5 shows positive immunoblots for embryonic MHC in COPD and control diaphragms. However, at this level of protein loading (i.e., 20 µg/lane for each of the 3 lanes), our SDS-PAGE did not resolve the MHC band into its individual components (e.g., slow, IIa, developmental, and so forth). Moreover, because the developmental MHCs were present in such low concentrations, we exposed lanes 2 and
Fig. 6. Results of RT-PCR in a representative control and a representative COPD diaphragm. MHC transcripts appear above each pair of columns. For each transcript, left lane (i.e., odd numbers) shows control samples, and right lane (i.e., even numbers) contains COPD specimen. Lane S, molecular weight markers at increments of 100-kb pairs.

3 for an appreciably longer time than lane 1; nonetheless, Fig. 5 shows that lane 1 (stained with NOQ7.5.4D for slow MHC) was still appreciably darker, reflecting a greater concentration of slow MHC in the diaphragm. Additionally, we must consider the possibility that, at this high concentration of protein, our embryonic MHC antibody (i.e., F4–2B6) may have been reacting with some MHC or other muscle protein. However, extensive testing over the past 15 years (by N. Rubinstein) has demonstrated that the F4–2B6 antibody does not cross-react with other MHCs or any other 200-kDa muscle protein.

RT-PCR Measurements of mRNA

Figure 6 shows results from a representative control and a representative COPD diaphragm. Figure 6 shows that both diaphragms contain PCR products consistent with the number of base pairs expected for RT-PCR of MHC IIb, the expected product was not obtained.

Because the presence of mRNA coding for embryonic MHC was important for a proper interpretation of our data, we isolated these PCR fragments from the gel,
cloned them into pGem-T vectors for amplification, and then sequenced them with an automatic sequencer. The 231-base fragment is homologous to a region encompassing exons 39 and 40 of the gene coding for embryonic mRNA (5′-ACATGACTGGAGGAACGTG CGGATATCGCGAGAATCCTAAA GTCAACAAAGTCCGGCTAA GACTCGAGGCATCCCTCCA GGA-

**DISCUSSION**

Summary of Major Findings

First, using isoform-specific antibodies, we demonstrate the occurrence of developmental (i.e., embryonic and neonatal) MHCs in appreciable proportions of fibers in control and COPD diaphragms; to the best of our knowledge, this constitutes the first systematic demonstration of this phenomenon in the adult human diaphragm. Second, we show that these developmental MHCs occurred predominantly in diaphragmatic fibers expressing MHC IIa. Third, we point out that these developmental MHCs represent such a small fraction of the MHC content of the diaphragm that they cannot be demonstrated by conventional SDS-PAGE techniques. Fourth, we confirm our previous observation that the adult human diaphragm adapts to severe COPD by an increase in the proportion of fibers expressing slow MHC and a decrease in the proportion of fibers expressing fast MHCs (24).

Critique

Developmental MHCs. Our previous experience, and that of others, indicates that antibodies F4–2B6 (10) and BF–34 (S. Schiaffino, personal communication) are specific for embryonic and neonatal MHCs, respectively. Therefore, we interpret our immunocytochemical results (Figs. 1–3) as indicating that these developmental MHCs are expressed in the adult human diaphragms of patients with severe COPD as well as controls. Moreover, our RT-PCR finding of mRNA coding for these MHCs supports our finding that these developmental MHCs are expressed in the adult human diaphragm.

The fact that we had to increase the amount of protein loaded per lane in our SDS-PAGE experiments by 10-fold to demonstrate the embryonic MHC suggests that this MHC accounts for a very small proportion of the MHC content of the adult human diaphragm. However, this finding probably does not represent cross-reaction with the other MHCs, because our original report (10) on the F4–2B6 antibody showed its specificity in human muscle as well as rodent muscle. Therefore, the combination of the SDS-PAGE and immunocytochemical results for embryonic MHC suggests that the very small amounts of embryonic MHC in the adult diaphragm (i.e., the SDS-PAGE finding) are distributed over a large proportion of the diaphragmatic fibers (the immunocytochemical finding).

**Is MHC IIx or IIb expressed in the human diaphragm?** Much work regarding adaptations of the diaphragm to COPD has been carried out in rats and hamsters, which have diaphragms that contain a third fast MHC, designated IIx (21, 45, 50). At the present time, an antibody that specifically stains fibers expressing this MHC does not exist. Rather, the only method for immunocytochemical detection of this MHC is via the negative antibody BF-35 that stains all fibers except those expressing only MHC IIx (45). Because all the fibers in all diaphragms that we studied were positive for at least one of the adult MHCs (i.e., I and IIa), our immunocytochemical findings can be interpreted as indicating that no fibers containing pure MHC IIx were noted in COPD or control diaphragms.

Recently, on the basis of in situ hybridization techniques for mRNA coding for MHCs, Smerdu et al. (47) concluded that human muscle contained three principal fiber types (i.e., I, IIa, and IIx), expressing a single MHC transcript, and two populations of hybrid fibers, coexpressing /slow and IIa or IIa and IIx MHC transcripts. These authors suggested that MHC IIb may not be expressed in human muscle, even though an MHC IIb gene does exist in this species (Shrager, unpublished observations). In our RT-PCR experiments, we recovered the anticipated PCR product for mRNA coding for MHC IIx but not for MHC IIb. Therefore, our RT-PCR results are consistent with the conclusions of Smerdu et al. as well as with other investigators (8, 34). However, our experimental data do not directly address the question of whether MHC IIb or MHC IIx is the major fast MHC, other than IIa, in the adult human diaphragm. Nonetheless, because we had to amplify the BF-F3 reaction in our immunocytochemical studies, the possibility exists that BF-F3, in these experiments, was cross-reacting with an MHC other than IIb. Because our immunohistochemical results are not consistent with BF-F3 cross-reacting with slow, IIa, embryonic, or neonatal MHCs, the possibility exists that (in these experiments) BF-F3 was reacting with MHC IIx. Accordingly, in all subsequent references to these BF-F3-reactive fibers, we use the term “IIb/x” to refer to these fibers.

**MHC coexpression patterns.** Our experiments with the subset of diaphragms that were stained with the BF-F3 antibody indicated that virtually all the fibers containing IIa, embryonic, and neonatal MHCs in COPD and control diaphragms (Fig. 1) also demonstrated BF-F3 reactivity, which we will interpret as indicating that these fibers also contained MHC IIb/x. Accordingly, we interpret the data in Fig. 3 as indicating that the adult human diaphragm (in patients with severe COPD and controls) contains two basic fiber types: a slow type consisting of pure type I fibers and a fast type consisting of fibers containing IIa, embryonic, neonatal, and IIb/x MHCs. The data in Fig. 3 indicate that >87% of the fibers in severe COPD and control diaphragms is accounted for by these two fiber types.
Comparison of Our Results With the Literature

Situations other than COPD in which developmental MHCs are expressed in the adult human diaphragm. In adult humans, previous work demonstrated that certain "special muscles," such as the extraocular (52) and masseter muscles (46, 48), contain developmental MHCs. However, in these special muscles, developmental MHCs are present in much larger concentrations, and they can be demonstrated by conventional electrophoretic techniques, such as SDS-PAGE and native gel electrophoresis. Other than these special muscles, systematic studies have also demonstrated these developmental MHCs in various muscular diseases exhibiting fiber regeneration, fiber degeneration, and denervation of fibers (e.g., muscular dystrophies and polymyositis) (9, 12), transplanted muscle (53), and a subset of patients with dilated cardiomyopathy (26). In all these circumstances, developmental MHCs are present in muscles exhibiting such pathological features as fibrosis, myocyte necrosis, and predominance of central nuclei. However, Fig. 7 demonstrates that, in our COPD and control diaphragms, these developmental MHCs occurred in microscopically normal muscle. Additionally, to detect more subtle changes of muscle injury, we stained these sections with desmin and fibronectin, as previously described by others (25). We noted that all the muscle fibers stained positively for desmin and fibronectin, as previously described by others (25). We noted that all the muscle fibers stained positively for desmin and fibronectin, as previously described by others (25). We noted that all the muscle fibers stained positively for desmin and fibronectin, as previously described by others (25). We noted that all the muscle fibers stained positively for desmin and fibronectin, as previously described by others (25).

Adaptation of the adult human diaphragm to COPD. The data of Sanchez et al. (41, 42) indicate that moderate COPD is associated with atrophy of type I and II fibers without any change in relative proportions of these fiber types. However, our COPD patients had more severe disease manifest by greater abnormalities in spirometric indexes and appreciably greater hyperinflation. This latter comparison suggests that only the diaphragms of patients with severe COPD exhibit the transformation of fast- to slow-twitch fiber type exhibited by our COPD diaphragms. The immunocytochemical results of the present study are consistent with our recently published MHC electrophoretic data (24), as well as that of Mercadier et al. (30); both studies demonstrated that a transformation from fast to slow diaphragm myosin isoforms is associated with severe COPD. Additionally, our previous work indicated that severe COPD was also associated with transformation from fast to slow nonmyosin myofilament proteins, such as tropomyosin and the troponin subunits (24).

Possible Explanations for Our Finding of Developmental MHCs in the Adult Diaphragm

First, the possibility exists that these developmental MHCs are related to the adaptations elicited by severe COPD in the diaphragm. For example, during the transition from fast- to slow-twitch fibers, the fast-twitch fibers in the diaphragm may temporarily express these developmental MHCs (29). However, we noted that embryonic and neonatal MHCs occur in a greater proportion of fibers in the normal diaphragm than in the COPD diaphragm. Therefore, we do not believe that this explanation is tenable.

Second, developmental MHCs differ from adult MHCs with respect to maximum isometric force, velocity of unloaded shortening, and actomyosin ATPase activity (14). Therefore, the possibility exists that the diaphragm, the only skeletal muscle that is constantly active, may require these developmental MHCs to carry out its unique physiological role. Although we cannot eliminate this possibility, we do not believe that this is the case, because we noted similar "trace" expression of these developmental MHCs in preliminary experiments with respiratory muscles other than the diaphragm (i.e., paraesternal intercostals) as well as limb muscles (i.e., gastrocnemius).

Third, previous investigators have demonstrated that the genes for type IIa, embryonic, neonatal, and type I1b MHCs lie in close proximity on chromosome 17 (7, 11, 22, 37, 49). Therefore, we believe that the most likely explanation for the trace amounts of embryonic and neonatal MHCs noted in COPD and control diaphragms is some nonspecificity in the transcription and regulation of mRNA codings for these different MHCs. Some workers in this field use the term "leakiness of transcription" to refer to this hypothesized phenomenon.

In conclusion, we point out that the gene for slow MHC lies on chromosome 14 (11, 40). Therefore, the above-noted hypothetical nonspecificity of transcription would not explain the coexpression of slow MHC (i.e., type I) with any of the fast or developmental MHCs. However, Fig. 3 shows that the proportion of these type fibers is quite small, and the possibility exists that these fibers may represent fibers undergoing transformation.

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