Relationship between Utrophin and Regenerating Muscle Fibers in Duchenne Muscular Dystrophy

Thesis by

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Relationship between Utrophin and Regenerating Muscle Fibers in Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is the most common and most severe form of muscular dystrophy. Mutation of the dystrophin gene, which is located on the X chromosome at the p21 region, has been revealed to be responsible, which results in the absence of dystrophin in skeletal muscle fibers. Utrophin is a recently identified structural homologue of dystrophin. It is a 395 kD protein encoded by a gene located on chromosome 6q24. While dystrophin is absent, utrophin is reported to be up-regulated anomalously at the sarcolemma in DMD.

In order to understand the role of utrophin, the expression of dystrophin and utrophin was analysed in 17 cases of DMD, 3 cases of polymyositis and 1 case of dermatomyositis. Vimentin and desmin were used to identify regenerating muscle fibers to clarify the association of utrophin with muscle regeneration. The muscle fibers were also divided into three stages according to diameter and the expression pattern as well as the staining intensity of utrophin, vimentin and desmin according to these stages were analysed and compared. The following results were obtained.

1) Dystrophin was totally negative in most cases of DMD while all cases of

inflammatory myopathy (IM) were positive.

2) Utrophin was positive in 94.0% of DMD and 75.0% of IM. Utrophin was more frequently positive in muscle fibers from DMD, staining 36.4% of the entire muscle fibers as compared to 10.5% in IM ($p=0.001$). Utrophin-positive fibers were most commonly present in stage 1, measuring less than 30μm. Their frequency decreased with increase in stage, ie. diameter of muscle fiber ($p=0.001$, 0.013). Optical density was higher in small regenerating fibers.

3) Vimentin and desmin were positive in regenerating fibers in all cases of DMD and IM. They were more frequently positive in muscle fibers from DMD, staining 34.4% of the entire muscle fibers for vimentin and 35.4% for desmin, as compared to 21.8% for vimentin and 20.9% for desmin in IM ($p=0.001$, 0.001). Vimentin and desmin-positive fibers were most commonly present in stage 1, measuring less than 30μm. Their frequency decreased with increase in stage, ie. diameter of muscle fiber ($p=0.001$, 0.001). In DMD, optical density was also highest in stage 1 and decreased with increase in stage, ie. diameter of muscle fiber.

The above results show that utrophin, as well as vimentin and desmin, were most frequently positive in small muscle fibers in the early stage of regeneration. Small regenerating fibers also tended to stain more strongly. These fibers corresponded to the regenerating muscle fibers observed by hematoxylin-eosin stain. It can therefore be concluded that utrophin up-regulation in DMD is a regeneration-associated event which disappears as the muscle fibers mature. This is also the case in IM in which a lesser amount of degeneration and regeneration of muscle fibers occurs.

Key Words: Duchenne muscular dystrophy, inflammatory myopathy, dystrophin, utrophin, muscle regeneration, vimentin, desmin

Relationship between Utrophin and Regenerating Fibers

in Duchenne Muscular Dystrophy

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Introduction

DMD is the most common form of muscular dystrophy, affecting about 1 in 3500 males. It is an X-linked disorder and presents with proximal weakness of the pelvic girdle muscles between 3 and 6 years of age. Progressive weakness of axial and appendicular muscles leads to loss of ability to ambulate in the early teens and eventually death in the late teens or early twenties due to respiratory or cardiac failure. DMD results from mutation of the dystrophin gene which is located in the Xp21 region.

Dystrophin, the protein product of the dystrophin gene, is a 427 kD cytoskeletal protein of the spectrin/α-actinin superfamily. It is expressed predominantly in skeletal, cardiac and smooth muscle[1,2]. In skeletal muscle, it is located mainly at the sarcolemma but also as a minor component at the neuromuscular junction and myotendinous junction. It is composed of three parts; the amino-terminus, rod domain and carboxyl-terminus[1,2]. The amino-terminus binds to actin in the cytoskeleton and the carboxyl-terminus binds to the dystrophin-associated protein complex, which is connected to the extracellular matrix[1-4]. Therefore dystrophin is thought to act as a link between the cytoskeleton of the muscle fiber and the extracellular matrix and thus functions to maintain the integrity of the cell membrane during repeated cycles of muscle contraction and

relaxation[4-7]. Some of the other functions dystrophin is suggested to perform include mediation of signals between the cytoskeleton and the extracellular matrix, force transduction from the cytoskeleton to the extracellular matrix and organization of membrane specializations[3,8]. The rod domain is composed of 24 repeating units of 110 amino acids, interrupted by 4 hinge regions. Deletions in the amino-terminus results in various phenotypes ranging from mild to severe and deletions in the central rod domain give rise to Becker muscular dystrophy[2]. Meanwhile, deletions in the cysteine-rich domain and the first half of the carboxyl-terminus results almost always in DMD, suggesting that this is the most important region for the normal function of dystrophin[3].

Utrophin is an autosomal homologue of dystrophin with a molecular weight of 395 kD. It was first reported that fragments from the carboxyl-terminus of the DMD complementary deoxyribonucleic acid (cDNA) detected a closely related sequence which exhibited nucleic acid and predicted amino acid identities with dystrophin of approximately 65% and 80%, respectively[9]. Sequencing revealed the two proteins to be homologous along the entire length[10]. Utrophin has been called variously as B2, dystrophin-related protein and DMD-like protein[9-11]. The utrophin gene is located on chromosome 6q24[9]. The only known structural difference with dystrophin is that utrophin lacks repeats 15 and 19 and 2 hinge regions in the rod domain[4]. Utrophin is more widely distributed than dystrophin, being present in the lung, kidney, placenta, liver, spleen and brain as well as muscle[12,13]. In skeletal muscle, it is located mainly at the neuromuscular junctions, myotendinous junctions, blood vessels and nerves[13-16]. Utrophin also differs from dystrophin with respect to the pattern of expression in fetal developing muscle. The level of expression of dystrophin increases from week 9 of gestation up to birth[17]. Utrophin is first detected before week 11, increases from week 11 to 17 and then gradually decreases up to week 26[18]. It is expressed minimally thereafter. Utrophin has therefore been proposed to be the fetal isoform of dystrophin[1,2].

Muscle biopsy specimen from DMD patients show minimal evidence of dystrophin by both immunohistochemical stain and Western blot. While dystrophin is lacking, utrophin expression is up-regulated anomalously at the sarcolemma[14-16,18-26]. Although the exact mechanism of utrophin

up-regulation is yet to be elucidated, as utrophin shares many structural properties with dystrophin, it has been suggested that utrophin may be used as a therapeutic replacement in DMD patients[1,8,19,27-30]. Utrophin may be up-regulated in DMD to compensate for the absence of dystrophin[1,13,16,19,25,27]. Alternatively, utrophin may be up-regulated as a consequence of muscle regeneration, as utrophin expression is also reported to be increased in IM such as polymyositis and dermatomyositis[16,21,24,31]. It has also been suggested that dystrophin and utrophin compete for the sarcolemma and when dystrophin disappears in DMD, utrophin takes its place instead[24]. Class I major histocompatibility complex and heat-shock protein are reported to be expressed in many muscular diseases including DMD, polymyositis and dermatomyositis as a nonspecific stress reaction[16]. Utrophin up-regulation may be explained similarly[16].

Regeneration of skeletal muscle fibers is a process similar to the normal development of skeletal muscle and undergoes a process of activation of satellite cells to myoblasts that undergo commitment, differentiation and fusion to form primary and secondary myotubes and eventually grow into regenerating fibers[32]. Vimentin and desmin are intermediate filament proteins which in normal developing muscle fibers are expressed in myoblasts and myotubes but their reactivity decreases with maturation[33-37]. There have been sporadic reports that overexpression of these proteins are present in regenerating muscle and some developmental myopathies[31,38,-40].

In order to understand the role of utrophin, the expression patterns of dystrophin and utrophin in skeletal muscle from DMD patients were analysed by immunohistochemical staining. And to clarify the association of utrophin with muscle regeneration, regenerating muscle fibers were identified by immunohistochemical staining for vimentin and desmin. The muscle fibers were also divided into three stages according to diameter as measured by image analysis. The expression pattern as well as the staining intensity, which was also measured by image analysis, of utrophin, vimentin and desmin according to these stages were analysed and compared.

Materials & Methods

1. Materials

Twenty-two cases of DMD diagnosed at Yongdong Severance Hospital from between August, 1999 and April, 2000 were examined. The diagnosis of DMD was made on the basis of clinical features, skeletal muscle biopsy, immunohistochemical study and dystrophin gene analysis using the polymerase chain reaction method. Five cases in which the results of immunohistochemical staining results were difficult to assess due to severe loss of muscle fibers and severe degree of fibrosis were excluded. Three cases of polymyositis and 1 case of dermatomyositis diagnosed at Yonsei University Medical Center during the same period were also included for comparison.

2. Methods

A. Skeletal muscle biopsy

Skeletal muscle biopsies from the vastus lateralis in 8 cases, biceps in 2 cases, gastrocnemius in 2 cases, rectus lateralis in 2 cases, deltoid in 1 case, quadriceps in 1 case and from unknown sites in 6 cases were performed.

B. Histochemical stain

The biopsied skeletal muscle were immersed immediately in isopentane (2-methyo-butane) and snap frozen at -160 170℃. The frozen specimen were sectioned in 8㎛ slices with a cryostat and hematoxylin-eosin and modified Gomori trichrome stains were performed.

(1) Hematoxylin-eosin stain

Hematoxylin-eosin stain was performed using the routine method.

(2) Modified Gomori trichrome stain

Sections were stained with Harris hematoxylin for 2 minutes and rinsed with distilled water. After staining with Gomori trichrome for 10 minutes, sections were differentiated in 0.2% acetic acid for 30 seconds.

C. Enzyme histochemistry

Cryostat sections were stained for NADH-tetrzolium reductase (NADH-TR), adenosine triphosphatase (ATPase) pH 9.4, pH 4.6 and pH 4.3.

(1) NADH-TR

Sections were immersed in NADH-TR solution consisting of 10ml Gomori Tris buffer, 10mg nitroblue tetrazolium and 4mg NADH for 30 minutes, rinsed with distilled water and developed with 2% nuclear red.

(2) ATPase pH 9.4

pH 9.4 solution consisting of 3.75g glycerin, 2.925g NaCl, 11.03g $CaCl_2$ and 600ml distilled water was adjusted to pH 9.4 using 0.1N NaOH. Sections were incubated in 10ml of this solution mixed with 15mg ATPase in a 37℃ water bath for 15 minutes. After rinsing with distilled water, sections were immersed in 2% cobaltous chloride solution for 5 minutes. After rinsing with distilled water, sections were immersed in 2% ammonium sulfide .

(3) ATPase pH 4.6 and 4.3

Solution A consisting of 9.7g sodium acetate, 14.7g sodium barbital and 500ml distilled water and solution B consisting of 4.25ml HCl and 500ml distilled water were prepared.

For ATPase pH 4.6, sections were pre-incubated in pH 4.6 solution consisting of 10ml solution A, 20ml solution B and 16ml distilled water for 10 minutes. After rinsing with distilled water, the staining procedure for pH 9.4 as described was followed.

For ATPase pH 4.3, sections were pre-incubated in pH 4.3 solution consisting of 10ml solution A, 24ml solution B and 12ml distilled water for 10 minutes. After rinsing with distilled water, the staining procedure for pH 9.4 as described was followed.

D. Immunohistochemistry

Cryostat sections mounted on silane-coated slides were air-dried at room temperature for 30 minutes. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 for 30 minutes and rinsing with phosphate-buffered saline (PBS) for 10 minutes. Sections were then incubated overnight at 4℃ with primary antibodies. Monoclonal antibodies diluted 1:50 against dystrophin rod domain, carboxyl-terminus, amino-terminus (NCL-DYS 1, NCL-DYS 2, NCL-DYS

3, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), utrophin amino-terminus (NCL-DRP2, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), desmin (NCL-DES-DERII, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) and vimentin (NCL-VIM, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) were used. After rinsing with PBS, sections were incubated with biotin-labelled secondary antibodies using the DAKO LSAB kit (DAKO corporation, Carpinteria, CA, USA) at room temperature for 30 minutes. After rinsing with PBS, sections were incubated with streptavidin peroxidase at room temperature for 30 minutes. After rinsing with PBS, sections were developed using amino-ethyl carbazole (Bimeda Corp., Foster, CA, USA), rinsed with water and counterstained with hematoxylin.

For dystrophin and utrophin, sections showing staining along the entire circumference of the sarcolemma of indivisual muscle fibers were interpretated as positive. For vimentin and desmin, sections showing homogeneous staining of the entire cytoplasm of indivisual muscle fibers were interpretated as positive.

E. Image analysis

Sections stained with hematoxylin-eosin and immunohistochemistry were magnified to ×200 using a light microscope and the images were captured with a digital microscopic camera. The captured images were then analysed using an image analysis system equipped with Image-Pro plus version 3.0 (Media Cybernatics, Silver Spring, Maryland, USA).

(1) Measurement of diameter of muscle fibers and divison of muscle fibers into three stages

Diameter of muscle fibers was measured using image analysis. The diameter perpendicular to the maximal diameter was measured to overcome the distortion which occurs when a fiber is cut obliquely.

Muscle fibers were divided into three stages according to diameter. Muscle fibers measuring less than 30㎛ were defined as stage 1, those measuring 30㎛ or greater but less than 50㎛ as stage 2 and those measuring 50㎛ or greater were defined as stage 3.

(2) Measurement of optical density

Optical density of utrophin, vimentin and desmin immunohistochemistry results were measured using image analysis. A scale of 0-255 was used. Lower values indicated greater labelling with 0 indicating the darkest pixel and 255 indicating the lightest pixel. As the imaging software did not allow free-form tracing, a line was used to measure the optical density of utrophin and a circular field approximating the area of the indivisual muscle fiber was used for vimentin and

desmin.

F. Statistical analysis

Chi-square test using the SAS system was performed to evaluate whether the difference in frequency between DMD and IM with respect to utrophin, vimentin and desmin-positive fibers is statistically significant. Chi-square test for equal proportions using the SAS system was performed to evaluate whether there is a statistically significant relationship between utrophin, vimentin and desmin-positive fibers and stage, ie. diameter of muscle fiber in DMD and IM. A one-way analysis of variance (ANOVA) with pair-wise comparison was performed to evaluate whether there is a statistically significant relationship between optical density of utrophin, vimentin and desmin-positive fibers and stage, ie. diameter of muscle fiber in DMD and IM. The patient factor was controlled. The level of significance was set to $p<0.05$.

Results

1. Clinical findings

A. Duchenne muscular dystrophy

All of the 17 patients were male with a mean age of 7.6 years (range 1-16 years) (Table 1). Five of the 17 patients (29.4%) were confined to a wheelchair at the time of this study.

B. Inflammatory myopathy

All of the 4 patients with polymyositis and the 1 patient with dermatomyositis were female with a mean age of 44.8 years (range 35-55 years) (Table 1).

Table 1. Clinical characteristics of Duchenne muscular dystrophy and inflammatory myopathy

Patients	Number	Sex(M:F)	Mean age (Range)
Duchenne muscular dystrophy	17	17:0	7.6 (1-160)
Inflammatory myopathy	4	0:4	44.8 (35-55)
Polymyositis	3	0:3	41(38-51)
Dermatomyositis	1	1:0	55

2. Light microscopic findings

A. Duchenne muscular dystrophy

Variation in diameter of the muscle fibers was prominent with increased number of internalized nuclei (Fig. 1A). Degeneration and necrosis of muscle fibers as well as phagocytosis were noted alongside regenerating fibers. These fibers were of small size and were seen in small groups. They demonstrated basophilic cytoplasm, enlarged nuclei with vesicular chromatin pattern and prominent nucleoli. Endomysial fibrosis and fat infiltration were noted in advanced cases. Three of the 17 cases (17.6%) showed marked atrophy of muscle fibers with muscle fiber loss.

B. Inflammatory myopathy

Inflammatory cell infiltration was the primary feature of the muscle biopsies from polymyositis and dermatomyositis (Fig. 1B). The distribution of the infiltrate was interstitial in polymyositis, while it was perivascular in dermatomyositis. Necrosis as well as regeneration of muscle fibers were also present. Advanced cases demonstrated endomysial fibrosis, fat infiltration and muscle fiber loss.

3. Enzyme histochemistry findings

A. Duchenne muscular dystrophy

Type I fiber predominance was demonstrated in an average of 75% of the muscle fibers in 2 of the 17 cases (11.8%) using the ATPase pH 9.4. pH 4.6, pH 4.3 and NADH-TR stains. No grouped atrophy was present.

B. Inflammatory myopathy

ATPase pH 9.4. pH 4.6, pH 4.3 and NADH-TR stains demonstrated no fiber type predominance or grouped atrophy.

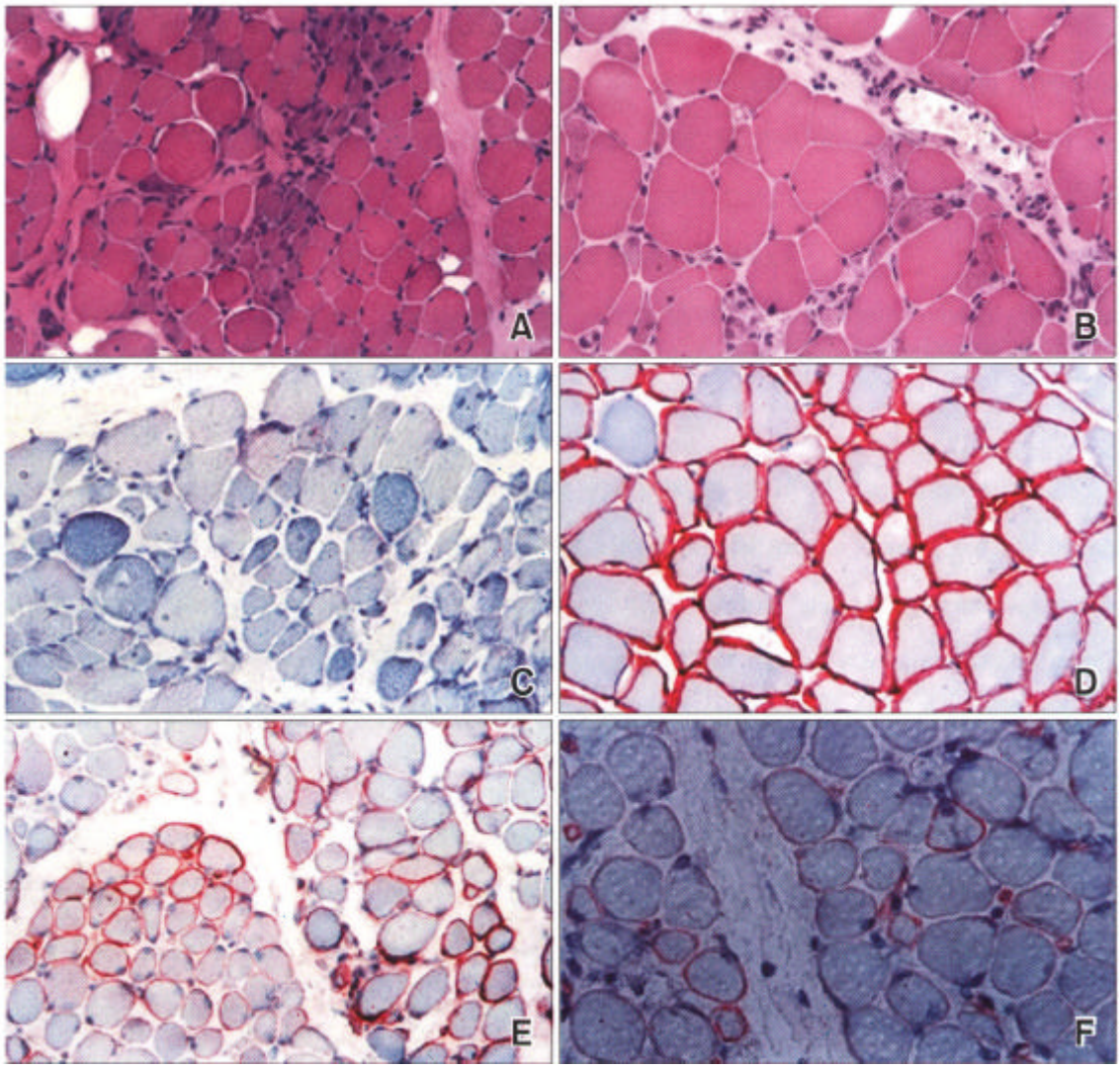

Fig. 1. Findings of hematoxylin-eosin stain (A, B) and immunohistochemical staining for dystrophin (C, D) and utrophin (E, F) in Duchenne muscular dystrophy (A, C, E) and inflammatory myopathy (B, D, F)

4. Immunohistochemistry findings

A. Dystrophin

(1) Duchenne muscular dystrophy

Dystrophin was completely negative in most cases of DMD (Fig. 1C). In 7 of the 17 cases (41.2%), a few of the muscle fibers demonstrated positive results.

Of these, 6 cases were positive for either dystrophin rod domain (NCL-DYS 1), carboxyl-terminus (NCL-DYS 2) or amino-terminus NCL-DYS 3), while 1 case was positive for both dystrophin rod domain (NCL-DYS 1) and amino-terminus (NCL-DYS 3). Rod domain (NCL-DYS 1) was positive in 2 of the 17 cases (11.6%), carboxyl-terminus (NCL-DYS 2) in 1 of the 17 cases (5.9%) and amino-terminus (NCL-DYS 3) in 5 of the 17 cases (29.4%).

(2) Inflammatory myopathy: Dystrophin was positive in all 3 cases of polymyositis and 1 case of dermatomyositis (Fig. 1D).

B. Utrophin

(1) Duchenne muscular dystrophy: Utrophin was positive in 16 of the 17 cases (94.0%) of DMD (Fig. 1E). Two hundred and eighty-four of a total of 780 muscle fibers (36.4%) obtained from the 16 cases demonstrated positive staining. One hundred and ninety-five of the 284 utrophin-positive muscle fibers (68.7%) were in stage 1, 82 of the 284 utrophin-positive muscle fibers (28.9%) in stage 2 and 7 of the 284 utrophin-positive muscle fibers (2.5%) in stage 3 (p=0.001) (Fig. 2A).

(2) Inflammatory myopathy: Utrophin was positive in 3 of the 4 cases (75.0%) of IM. Thirteen of a total of 124 muscle fibers (10.5%) obtained from the 3 cases demonstrated positive staining (Fig. 1F). Eleven of the 13 utrophin-positive muscle fibers (84.6%) were in stage 1 and 2 of the 13 utrophin-positive muscle fibers (15.4%) in stage 2. There were no fibers in stage 3 (p=0.013) (Fig. 2A).

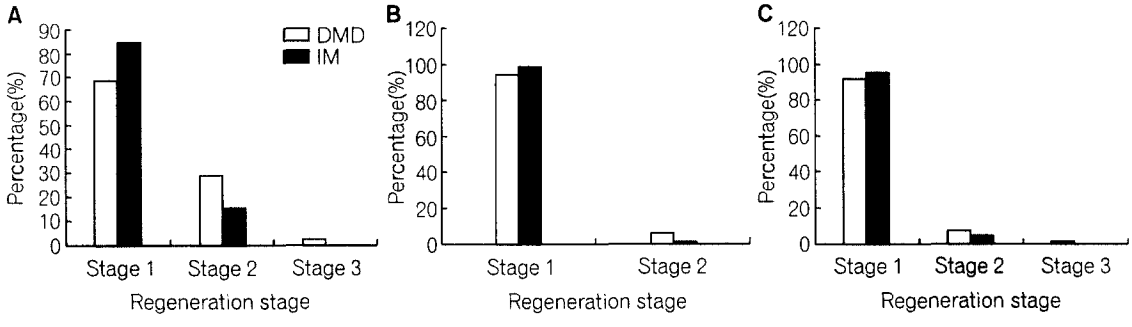

Fig. 2. Frequency of utrophin (A), vimentin (B) and desmin (C)-positive fibers in Duchenne muscular dystrophy and inflammatory myopathy.

C. Vimentin and desmin

Immunohistochemical stains for vimentin and desmin were performed in 15 of the 17 cases. Vimentin and desmin were positive in a variable number of muscle fibers in all of the cases of DMD and IM. Muscle fibers demonstrating positive staining were small and corresponded to regenerating fibers when compared with sections stained with hematoxylin-eosin.

The staining patterns of vimentin and desmin were variable, but in general, vimentin was more inhomogeneously stained and weaker in intensity than desmin. Desmin was more homogeneously stained and stronger in intensity than vimentin.

(1) Vimentin

(A) Duchenne muscular dystrophy; Vimentin was positive in 266 of a total of 774 muscle fibers (34.4%) obtained from the 15 cases demonstrating vimentin positivity in DMD (Fig. 3A). Two hundred and fifty of the 266 vimentin-positive muscle fibers (94.0%) were in stage 1 and 16 of the 266 vimentin-positive muscle fibers (6.0%) in stage 2. There were no fibers in stage 3 ($p=0.001$) (Fig. 2B).

(B) Inflammatory myopathy; Vimentin was positive in 74 of a total of 340 muscle fibers (21.8%) obtained from all 4 cases demonstrating vimentin positivity in IM (Fig. 3B). Seventy-three of the 74 vimentin-positive muscle fibers (98.6%) were in stage 1 and the remaining 1 of the 74 vimentin-positive muscle fibers (1.4%) in stage 2. There were no fibers in stage 3 ($p=0.001$) (Fig. 2B).

(2) Desmin

(A) Duchenne muscular dystrophy; Desmin was positive in 273 of a total of 771 muscle fibers (35.4%) obtained from the 15 cases demonstrating desmin positivity in DMD (Fig. 3C). Two hundred and fifty of the 273 desmin-positive muscle fibers (91.6%) were in stage 1, 20 of the 273 desmin-positive muscle fibers (7.3%) in stage 2 and 3 of the 273 desmin-positive muscle fibers (1.1%) in stage 3 ($p=0.001$) (Fig. 2C).

(B) Inflammatory myopathy; Desmin was positive in 66 of a total of 316 muscle fibers (20.9%)

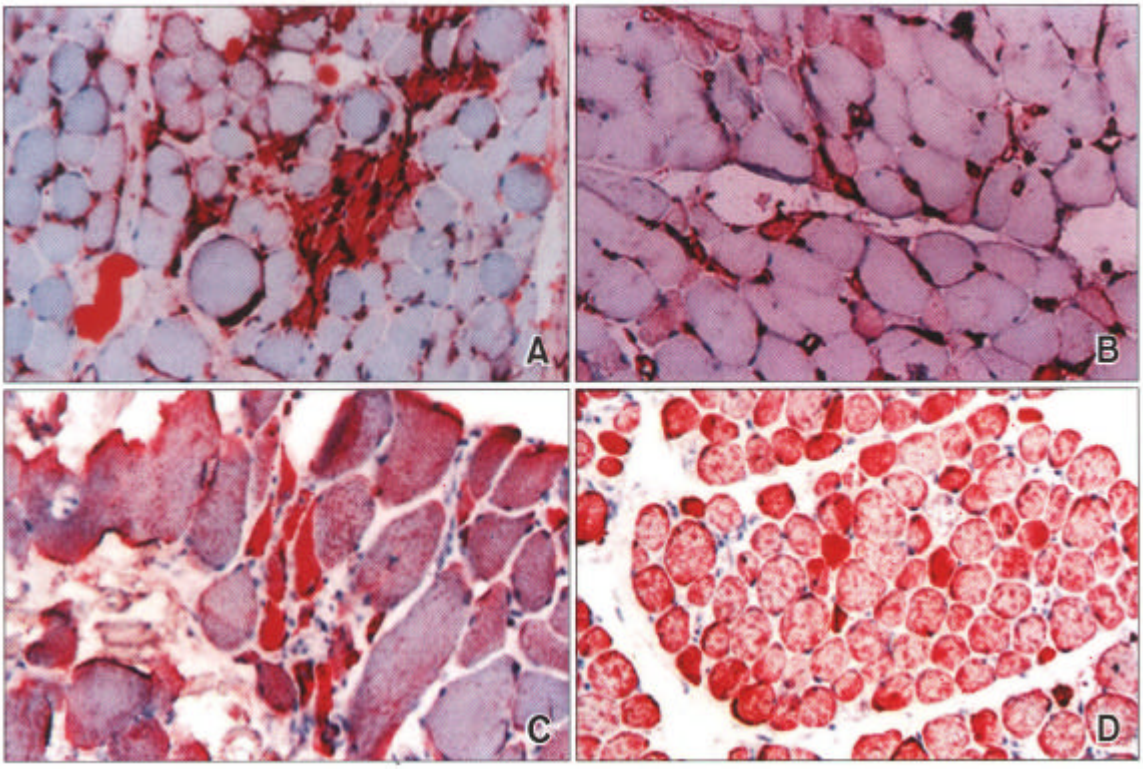

Fig. 3. Findings of immunohistochemical stains for vimentin (A, B) and desmin (C, D) in Duchenne muscular dystrophy (A, C) and inflammatory myopathy (B, D).

obtained from all 4 cases demonstrating desmin positivity in IM (Fig. 3D). Sixty-three of the 66 desmin-positive muscle fibers (95.5%) were in stage 1 and 3 of the 66 desmin-positive muscle fibers (4.5%) in stage 2. There were no fibers in stage 3 (p=0.001) (Fig. 2C).

5. Image analysis findings

A. Utrophin

(1) Duchenne muscular dystrophy: The mean diameter of utrophin-positive muscle fibers from the 16 cases demonstrating utrophin positivity in DMD was 18 μm (SD 7.3) in stage 1, 37.4 μm (SD 5.1) in stage 2 and 55.5 μm (SD 3.1) in stage 3 (Fig. 4A).

The mean optical density was 105.6 (SD 44.4) in stage 1, 113.3 (SD 48.3) in stage 2 and 123.5 (SD 56.0) in stage 3. The difference between stage 1 and stage 3, as well as between stage 2

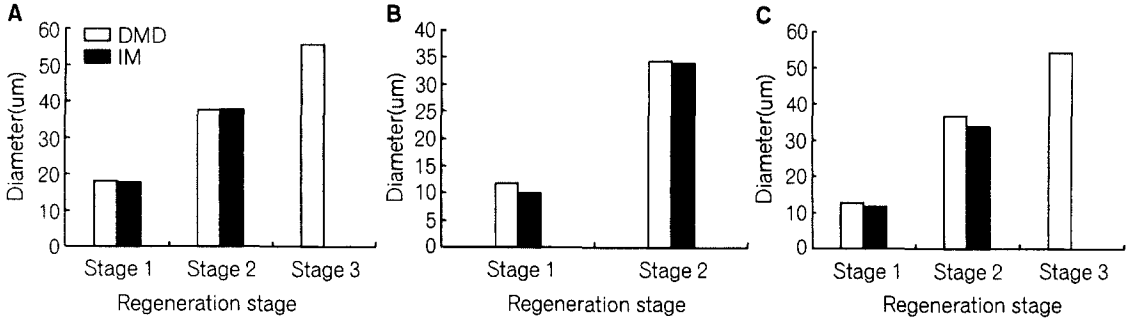

Fig. 4. Mean diameter of utrophin (A), vimentin (B) and desmin (C)-positive fibers in Duchenne muscular dystrophy and inflammatory myopathy according to regeneration stage.

and stage 3 was statistically significant (Fig. 5A, B, C, Table 2).

(2) Inflammatory myopathy: The mean diameter of utrophin-positive muscle fibers from the 3 cases demonstrating utrophin positivity in IM was 17.5 μm (SD 8.2) in stage 1 and 37.4 μm (SD 9.2) in stage 2. There were no fibers in stage 3 (Fig. 4A).

The mean optical density was 129.6 (SD 26.7) in stage 1, 146.0 (SD 15.0) in stage 2 and 136.6 (SD 12.8) in stage 3. There was a statistically significant difference between stage 1 and stage 2 (Table 3).

B. Vimentin

(1) Duchenne muscular dystrophy: The mean diameter of vimentin-positive muscle fibers from the 15 cases demonstrating vimentin positivity in DMD was 11.7 μm (SD 6.4) in stage 1 and 34.4 μm (SD 4.2) in stage 2. There were no muscle fibers in stage 3 (Fig. 4B).

The mean optical density was 97.8 (SD 56.3) in stage 1, 157.1 (SD 37.1) in stage 2 and 174.4 (SD 20.2) in stage 3. There was a statistically significant difference between the stages (Fig. 5D, E, F, Table 2).

(2) Inflammatory myopathy: The mean diameter of vimentin-positive muscle fibers from all 4 cases demonstrating vimentin positivity in IM was 9.9 μm (SD 4.7) in stage 1 and 34.0 μm in stage 2. There were no fibers in stage 3 (Fig. 4B).

The mean optical density was 115.9 (SD 43.4) in stage 1, 159.2 (SD 17.1) in stage 2 and 151.5

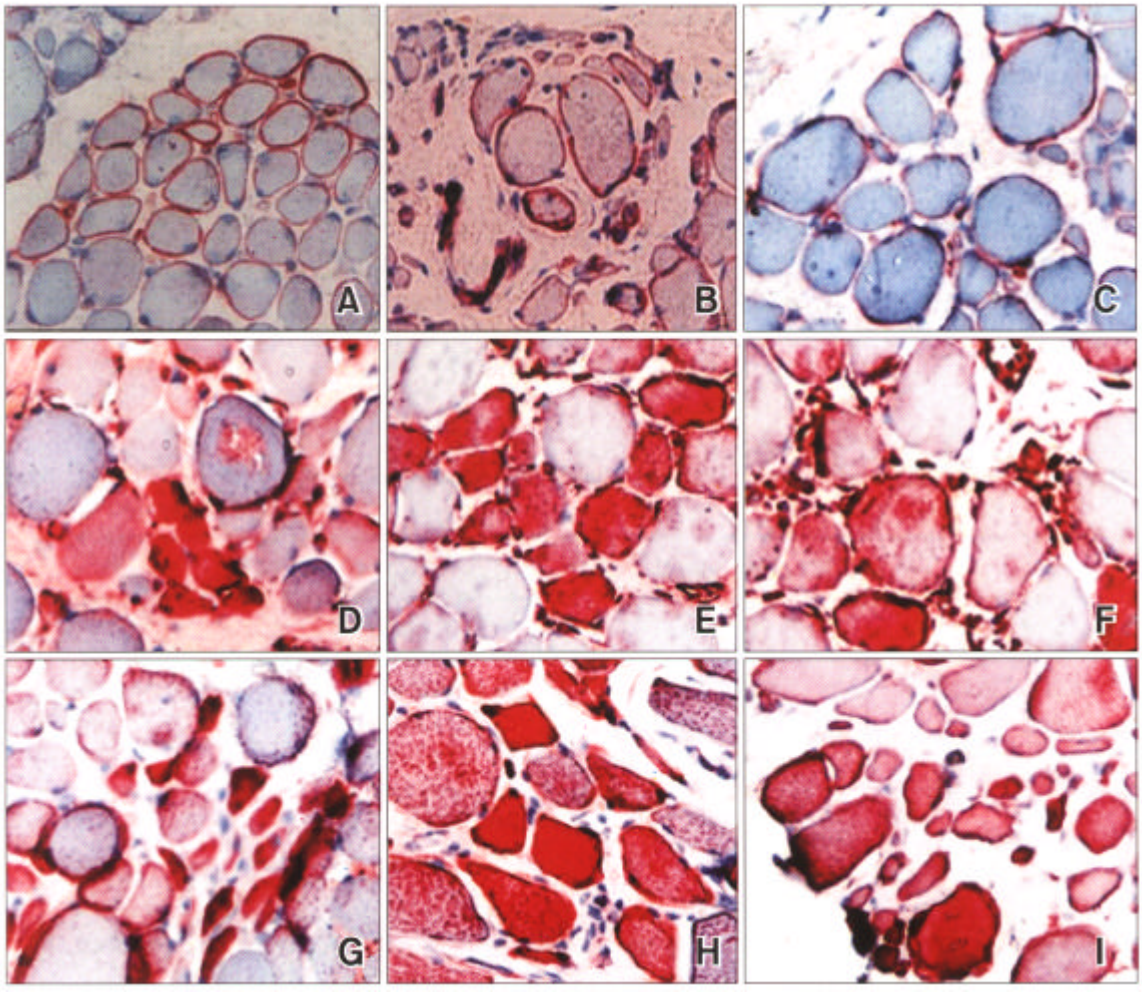

Fig. 5. Optical density of utrophin (A, B, C), vimentin (D, E, F) and desmin (G, H, I) in Duchenne muscular dystrophy according to regeneration stage (A, D, G: stage 1, B, E, H: stage 2, C, F, I: stage 3).

(SD 19.5) in stage 3. The difference between stage 1 and stage 2, as well as between stage 1 and stage 3 was statistically significant (Table 3).

C. Desmin

(1) Duchenne muscular dystrophy: The mean diameter of desmin-positive muscle fibers from the 15 cases demonstrating desmin positivity in DMD was 12.6 μm (SD 6.0) in stage 1, 36.5 μm (SD 4.0) in stage 2 and 54.2 μm (SD 2.6) in stage 3 (Fig. 4C).

Table 2. Optical density of utrophin, vimentin and desmin according to regeneration stage in Duchenne muscular dystrophy

Marker	Optical density (mean±SD)		
	Stage 1[1]	Stage 2	Stage 3
Utrophin	105.5±44.4	113.3±48.3	123.5±56.0
Vimentin	97.8±56.3	157.1±37.1	174.4±20.2
Desmin	87.5±49.2	129.9±44.9	151.5±39.1

[1]Stage 1: <30 μm, Stage 2: ≥30 μm, <50 μm, Stage 3; ≥50 μm

Table 3. Optical density of utrophin, vimentin and desmin according to regeneration stage in inflammatory myopathy

Marker	Optical density (mean±SD)		
	Stage 1[1]	Stage 2	Stage 3
Utrophin	129.6±26.7	146.0±15.0	136.6±12.8
Vimentin	115.9±43.4	159.2±17.1	151.5±19.5
Desmin	102.3±32.4	128.9±19.6	127.9±13.4

[1]Stage 1: <30 μm, Stage 2: ≥30 μm, <50 μm, Stage 3; ≥50 μm

The mean optical density was 87.5 (SD 49.2) in stage 1, 129.9 (SD 44.9) in stage 2 and 151.5 (SD 39.1) in stage 3. There was a statistically significant difference between the stages (Fig. 5, G, H, I, Table 2).

(2) Inflammatory myopathy: The mean diameter of desmin-positive muscle fibers from all 4 cases demonstrating desmin positivity in IM was 11.6 μm (SD 5.2) in stage 1 and 33.6 μm (SD 4.5) in stage 2. There were no fibers in stage 3 (Fig. 4C).

The mean optical density of desmin-positive fibers was 102.3 (SD 32.4) in stage 1, 128.9 (SD 19.6) in stage 2 and 127.9 (SD 13.4) in stage 3. The difference between stage 1 and stage 2, as well as between stage 1 and stage 3 was statistically significant (Table 3).

Discussion

Utrophin, encoded by the utrophin gene located on chromosome 6q24, is a structurally homologous protein of dystrophin. In normal adult skeletal muscle, utrophin is expressed only at the neuromuscular junctions, myotendinous junctions, blood vessels and nerves but it is overexpressed at the sarcolemma in DMD. Although information on the distribution and regulation of utrophin are becoming clear, knowledge on its function remains unknown. As utrophin is associated predominantly with the neuromuscular junction, it has been suggested that utrophin plays a role in synaptogenesis[1,2,25]. However, utrophin-deficient mice are reported to be healthy with no obvious signs of neuromuscular abnormality despite a decrease in the number of acetylcholine receptors as well as the number of postsynaptic membrane folds[41,42]. These results show that at the neuromuscular junction, utrophin is not essential during the early stage of synapse formation but is involved in the development and maintenance of the postsynaptic membrane. Because utrophin shares considerable sequence homology with dystrophin, many of the proteins that bind to dystrophin also bind to utrophin. The amino-terminus binds to actin[44] and the carboxyl-terminus binds to the dystrophin-associated protein complex[19], raising the possibility that utrophin may play a similar role as dystrophin at the sarcolemma.

Recently, it has been shown that utrophin is spontaneously up-regulated anomalously at the sarcolemma while dystrophin is lacking and the dystophin-associated protein level is reduced in DMD[14-16,18-26]. The mechanism for utrophin up-regulation is unclear as yet.

As utrophin and dystrophin are closely related, utrophin up-regulation in DMD may be a compensatory mechanism for the dystrophin deficiency[1,13,16,19,25,27]. In mdx mice, high levels of expression of a truncated utrophin transgene was shown to reduce the dystrophic pathology as demonstrated by decrease in the level of serum creatine kinase, reduced number of centralized nuclei and increase in dystrophin-related protein complex[28]. Furthermore, improvement in mechanical performance of muscle as demonstrated by recovery of force development, mechanical resistance to forced lengthening and maximal spontaneous activity as well as maintenance of

intracellular calcium homeostasis was also shown[29]. While mdx mice are physically normal despite the underlying pathology in skeletal muscle and mice deficient in utrophin also have a mild phenotype with subtle changes in the neuromuscular junction architecture[41,42], generation of mice deficient in both dystrophin and utrophin present with severe progressive muscular dystrophy resulting in premature death[43]. This difference suggests that utrophin and dystrophin play complementary roles in normal functional and developmental pathways in muscle and overexpression of one protein may compensate for the loss of the other one. But as mdx mice present with a milder disease than DMD in humans, direct comparison may be difficult. It has also been suggested that utrophin may compensate for dystrophin deficiency by retaining dystrophin-related proteins which are decreased in DMD, as utrophin and dystrophin-related proteins are found throughout the sarcolemma in small-caliber skeletal muscles and cardiac muscle of adult mdx mice, which are reported to show minimal pathological changes[19]. However, the up-regulated utrophin does not prevent muscle necrosis in DMD patients. And there is no relationship between the quantity and distribution of utrophin with the patient's age or severity of clinical course[45].

It has also been suggested that competition exists between dystrophin and utrophin for the sarcolemma[24]. When dystrophin is down-regulated, utrophin is up-regulated, taking the place of dystrophin.

However, utrophin is reported to be up-regulated not only in DMD but also in IM including polymyositis and dermatomyositis as well as in normal skeletal muscle[16,20,24,31]. Thus it has been argued that irrespective of dystrophin, utrophin is expressed in regenerating fibers. A recent study in hypertrophic feline muscular dystrophy demonstrated that utrophin is a regeneration-associated protein as it was transiently expressed in the early stage of regeneration and disappeared as the muscle fibers increased in size[31]. In the present study, utrophin was overexpressed in 94.1% of DMD patients and in 75.0% of IM patients. 36.4% of the muscle fibers were utrophin-positive in DMD patients while it was 10.5% in IM patients, reflecting that more muscle fibers in DMD undergo degeneration and regeneration than in IM.

It has previously been reported that the abnormal expression of utrophin in DMD and Becker

muscular dystrophy is age-related, being low in cases biopsied before 2 years of age and increased in those biopsied after 2 years of age[46]. As the utrophin-positive fibers were negative for fetal myosin, which is known to be present in fetal and regenerating fibers but not in mature muscle, it was suggested that abnormal expression of utrophin in mature muscle fibers in DMD and Becker muscular dystrophy is not a continuation of the expression that occurs in fetal or regenerating muscle, but is a secondary event caused by unknown factors.

Class I major histocompatibility complex protein products and heat-shock protein are reported to be positive in the sarcolemma in various muscular diseases including polymyositis, dermatomyositis and in some cases of DMD, while they are not expressed in normal muscle fibers[16]. Therefore utrophin up-regulation like these proteins may represent a nonspecific stress reaction.

It is of interest that although utrophin was overexpressed in DMD and polymyositis, there was no significant alteration in the level of utrophin messenger ribonucleic acids (mRNAs) between normal subjects as compared to DMD and polymyositis patients, suggesting that a post-transcriptional regulatory mechanism is responsible for the up-regulation[47]. Similar results were demonstrated in regenerating muscle in mice obtained by inducing severe muscle necrosis by injecting cardiotoxin.

Several different methods for the identification of regenerating skeletal muscle fibers have been described in the literature. The simplest method is by examination of the routinely studied hematoxylin-eosin stain. Regenerating muscle fibers are usually smaller than normal muscle fibers and appear grouped together. They have basophilic cytoplasm with internalized, enlarged, vesicular nuclei. In comparison, degenerating muscle fibers are pale-stained and are refered to as liquified or hyaline fibers. With progression of necrosis, the muscle fibers assume a round shape and are darkly eosinophilic and accompanied by phagocytosis. Other methods include staining with acridine orange for RNA[48], alkaline phosphatase[49], fetal myosin[46], vimentin and desmin[38-40]. There have been sporadic reports of vimentin and desmin expression in regenerating muscle and developmental myopathies. The rationale behind expression of vimentin and desmin in regenerating muscle fibers lies in that regenerating muscle fibers recapitulate normal myogenesis

including the reappearance of strong immunoreactivity for vimentin and desmin[31,38-40]. It was noted that regenerating muscle fibers in mice after induction of necrosis by injection of Ringer solution demonstrated positivity to vimentin and desmin[39]. Desmin reactivity persisted but vimentin disappeared after about 2 weeks. In the present study, vimentin and desmin were chosen to recognize regenerating fibers more precisely, as desmin was previously reported to stain regenerating fibers at a very early stage[38], while vimentin positivity was reported to be specific for regenerating muscle fibers with negative staining in mature fibers[39]. In normal muscle fibers, vimentin was non-reactive except at the blood vessels while desmin was lightly stained in a mosaic pattern. All cases of DMD and IM demonstrated positive staining for vimentin and desmin in small, regenerating muscle fibers. Overall, the staining pattern of vimentin and desmin demonstrated that vimentin was more inhomogeneously stained in the cytoplasm and weaker in intensity than desmin, while desmin more homogeneously stained and stronger in intensity than vimentin.

In the present study, the muscle fibers were divided into three stages as follows: stage 1 for those measuring less than 30μm, stage 2 for those measuring 30μm or greater and less than 50μm and stage 3 for those measuring 50μm or greater. When the expression patterns of utrophin, vimentin and desmin were compared according to the three stages, similar results were obtained. Most of the utrophin, vimentin and desmin-positive fibers were small ones in stage 1, thus representing regenerating fibers, in both DMD and IM. The frequency of positive fibers decreased with increase in fiber diameter and the difference between the stages was statistically significant. In DMD, the frequency of utrophin, vimentin and desmin-negative fibers measuring less than 30μm were 63%, 51% and 98%, respectively while in IM, it was 83%, 53% and 69%, respectively. It can be deduced from these results that these small fibers which were not labelled by utrophin, vimentin and desmin may represent muscle fibers undergoing degeneration or necrosis.

Semiquantitative analysis of the level of expression of utrophin has been performed previously[31] as well as measurement of intensity of immunoblot by image analysis[16], but this is the first study using image analysis to measure the optical density of utrophin, vimentin and

desmin using immunohistochemical staining, which was performed to objectively quantify the difference in staining intensity between the three stages. A line was used to measure the optical density of utrophin-positive fibers, as utrophin stains in a membranous pattern along the sarcolemma of the indivisual muscle fiber. A circular field approximating the area of the indivisual muscle fiber was used to measure the optical density of vimentin and desmin-positive fibers, as these show a cytoplasmic staining pattern. A scale of 0-255 was used, with 0 representing the darkest pixel and 255 representing the lightest pixel. The cut-off value used to define a positive stain was approximately 100. A pitfall in the measurement of optical density using immunohistochemical staining was that there was a slight variation in the staining quality between cases. Also, if the software system of image analysis could allow free-form tracing, a more exact measurement of optical density would be obtained. In DMD, there was a statistically significant difference in optical density of utrophin between stage 1 and stage 3, as well as between stage 2 and stage 3. In IM, there was a statistically significant difference between stage 1 and stage 2. For both vimentin and desmin, there was a statistically significant difference in optical density between the three stages in DMD. In IM, there was a significant difference between stage 1 and stage 2, as well as between stage 1 and stage 3. One of the factors responsible for the variable results of optical density is that the frequency of muscle fibers in each stage was uneven in distribution. Overall, the smaller fibers tended to be more intensely stained.

The fact that utrophin was expressed in both DMD and IM patients, that it was most frequently as well as more intensely expressed in the early stage of regeneration and that it resembles the expression patterns of vimentin and desmin suggest that the up-regulation of utrophin in DMD is associated with regeneration of skeletal muscle fibers, irrespective of dystrophin. The number of utrophin-positive fibers and the staining intensity decreased as the size of muscle fibers increased.

Current approaches at the treatment of DMD include replacement of dystrophin by myoblast transfer, gene replacement therapy and drug therapy. An alternative choice is the replacement of utrophin[1,8,19,27-30]. Utrophin offers the advantage that the utrophin gene is intact in DMD and that

utrophin is already exposed to the immune system. If the duration of utrophin expression can be sustained and its action potentiated, it may slow down the process of skeletal muscle necrosis in DMD patients.

Conclusions

In the present study, the expression patterns of dystrophin and utrophin were compared in skeletal muscle biopsy specimen obtained from 17 cases of DMD, 3 cases of polymyositis and 1 case of dermatomyositis. Muscle fibers were divided into three stages according to the diameter and the expression patterns of utrophin, as well as vimentin and desmin, which are known to be markers of regeneration, were compared according to stage. The relationship between optical density and stage was also analysed. The following results were obtained.

1) Dystrophin was totally negative in most cases of DMD, while all cases of IM were positive.

2) Utrophin was more frequently positive in muscle fibers from DMD than IM ($p=0.001$). Utrophin-positive fibers were most commonly present in stage 1, measuring less than 30㎛, and their frequency decreased with increase in stage, ie. diameter of muscle fiber ($p=0.001$, 0.001). Optical density was higher in smaller regenerating fibers.

3) Vimentin and desmin were more frequently positive in muscle fibers from DMD than IM ($p=0.001$, 0.001). Vimentin and desmin-positive fibers were most commonly present in stage 1, measuring less than 30㎛ and their frequency decreased with increase in stage, ie. diameter of muscle fiber ($p=0.001$, 0.001). In DMD, optical density was also highest in stage 1 and decreased with increase in stage, ie. diameter of muscle fiber.

The above results show that utrophin, as well as vimentin and desmin, were most frequently

positive in small muscle fibers in the early stage of regeneration. Small regenerating fibers also tended to stain more strongly. These fibers corresponded to the regenerating muscle fibers observed by hematoxylin-eosin stain. It can therefore be concluded that utrophin up-regulation in DMD is a regeneration- associated event and disappears as the muscle fibers mature. This is also the case in IM in which a lesser amount of degeneration and regeneration of muscle fibers occur.

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국문요약

뒤시엔느 근이영양증에서 재생근섬유와 유트로핀의 관계

뒤시엔느 근이영양증은 가장 심하고 가장 흔한 근이영양증으로 Xp21에 위치하는 뒤시엔느 근이영양증 유전자의 돌연변이에 의해 초래된다. Dystrophin은 뒤시엔느 근이영양증 유전자의 단백질 산물이고 뒤시엔느 근이영양증 환자의 골격근 조직에서 결여되어 있다. Utrophin은 최근에 발견된 dystrophin과 구조적으로 유사한 395 kD의 단백으로 6q24에 위치한 유전자에 의해 만들어진다. 뒤시엔느 근이영양증에서는 dystrophin의 발현이 결여되어 있는 반면 utrophin은 발현이 증가하고 정상 골격근에서와 달리 근섬유초에서 나타난다. 이와 같이 뒤시엔느 근이영양증에서 utrophin의 발현이 증가하는 기전에 대하여 아직 정확하게 알려져 있지 않다.

본 연구에서는 utrophin의 역할을 규명하기 위하여 뒤시엔느 근이영양증 17예, 다발성근염 3예와 피부근염 1예를 포함한 총 21예의 골격근 생검 조직에서 dystrophin과 utrophin의 발현 양상을 비교하였다. 또한 근섬유 재생과의 연관성을 자세히 알아보기 위하여 이들 질환에서 재생 근섬유의 표지자로 생각되는 vimentin과 desmin의 발현 양상을 보았고 utrophin의 발현 양상과 비교하였다. 그리고 근섬유를 직경에 따라서 3단계로 나누어 이에 따른 utrophin, vimentin과 desmin의 발현 양상과 optical density를 비교 분석하였으며 결과는 다음과 같았다.

1) Dystrophin은 뒤시엔느 근이영양증에서 대부분의 근섬유에서 음성 반응을 나타냈으며 염증성 근병증에서는 전 예에서 양성 반응을 나타냈다.

2) Utrophin은 뒤시엔느 근이영양증의 94.0%(16/17)와 염증성 근병증의 75.0%(3/4)에서 양성 반응을 나타냈다. 뒤시엔느 근이영양증에서는 전체 근섬유중 36.4%가 양성이었고

염증성 근병증에서는 10.5%가 양성으로 나타나서 뒤시엔느 근이영양증에서 훨씬 흔한 빈도에서 양성 반응을 나타냈다(p=0.001). Utrophin 양성 근섬유는 근섬유의 직경이 작을수록, 즉 초기 재생단계일수록 훨씬 흔한 빈도에서 나타났다(p=0.001, 0.013). Optical density는 근섬유의 직경이 작은 재생 근섬유에서 강하게 나타났다.

3) Vimentin과 desmin은 뒤시엔느 근이영양증과 염증성 근병증의 모든 예에서 양성 반응을 나타냈다. 뒤시엔느 근이영양증에서는 vimentin의 경우 전체 근섬유중 34.4%가 양성이었고 desmin은 35.4%가 양성으로 나타났으며 염증성 근병증에서는 vimentin의 경우 21.8%가 양성이었고 desmin의 경우 20.9%가 양성으로 나타나서 뒤시엔느 근이영양증에서 훨씬 흔한 빈도에서 양성 반응을 나타냈다(p=0.001). Vimentin과 desmin 양성 근섬유는 근섬유의 직경이 작을수록, 즉 초기 재생단계일수록 훨씬 흔한 빈도에서 나타났다(p=0.001, 0.001). Optical density는 뒤시엔느 근이영양증에서는 근섬유의 직경이 작을수록, 즉 초기 재생단계일수록 강하게 나타났다.

이상의 결과로 utrophin은 뒤시엔느 근이영양증과 염증성 근병증 모두에서 발현되고 vimentin과 desmin과 마찬가지로 근섬유 직경이 30㎛ 미만에 해당하는 초기 재생단계에서 대다수가 양성 반응을 나타내므로 뒤시엔느 근이영양증에서 utrophin의 발현이 증가하는 것은 근섬유 재생과 연관된 것으로 생각된다. 즉, 뒤시엔느 근이영양증에서 dystrophin의 발현이 결여되고 근섬유가 파괴되었다가 다시 재생되는 단계의 초기에 utrophin의 발현이 일시적으로 나타나는 것으로 생각된다.

핵심되는말: 뒤시엔느 근이영양증, 염증성 근병증, dystrophin, utrophin, vimentin, desmin, 근섬유 재생