Yonsei Medical Journal
Vol. 44, No. 1, pp. 15 - 23, 2003

Relationship between Utrophin and Regenerating Muscle Fibers in Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a dystrophinopathy, and its associated gene is located on Xp21. Moreover, utrophin, a recently identified structural homologue of dystrophin is reported to be up-regulated in DMD.

In order to investigate the association between utrophin and muscle regeneration in DMD, an immunohistochemical study using antibodies to utrophin, dystrophin, vimentin and desmin was carried out in 17 cases of DMD, 3 cases of polymyositis and 1 case of dermatomyositis.

Dystrophin was negative in almost all cases of DMD, but positive in all cases of inflammatory myopathy (IM). Utrophin was positive in 94.0% of DMD and in 75.0% of IM. 36.4% of the myofibers were positive in DMD, as compared to 10.5% in IM (p=0.001). In both groups, utrophin positivity was present most commonly in small regenerating fibers (p=0.001, 0.013). Vimentin and desmin were intensely positive in regenerating fibers in all cases of DMD and IM. 34.4% and 35.4% of myofibers were positive for vimentin and desmin in DMD, as compared to 21.8% and 20.9% in IM (p=0.001, 0.001). In both groups, vimentin and desmin positivity were present most commonly in small regenerating fibers (p=0.001, 0.001). The staining intensities of utrophin, vimentin and desmin were also higher in small regenerating fibers.

These results show that utrophin up-regulation is regeneration-associated, and that it is proportional to the quantity of regenerating myofibers, but is not specific for DMD.

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

Received January 18, 2002
Accepted October 23, 2002

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INTRODUCTION

DMD is the most common form of muscular dystrophy, affecting about 1 in 3500 males. It results from mutation of the dystrophin gene, which is located in the Xp21 region. Dystrophin is a 427 kD cytoskeletal protein expressed predominantly in skeletal, cardiac and smooth muscle.[1,2] In normal adult skeletal muscle, it is located mainly at the sarcolemma, but is also present as a minor component at the neuromuscular junction and myotendinous junction.

Utrophin is an autosomal homologue of dystrophin with a molecular weight of 395 kD, and its gene is located on chromosome 6q24.[3] It is more widely distributed than dystrophin, being present in the lung, kidney, placenta, liver, spleen and brain as well as muscle.[4,5] In normal adult skeletal muscle, it is located mainly at the neuromuscular and myotendinous junctions, and in the blood vessels and nerves.[5-8] Utrophin also differs from dystrophin with respect to the pattern of expression in fetal developing muscle.[9]

While dystrophin is lacking in DMD, utrophin expression is up-regulated anomalously at the sarcolemma.[6-8,10-18] Although the exact mechanism of utrophin up-regulation has 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.[1,11,19-24] Utrophin may be up-regulated in DMD to compensate for the absence of dystrophin.[1,5,8,11,17,19,24] 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 in

polymyositis and dermatomyositis.[8,13,16,25]

To investigate the role of utrophin in DMD and to clarify its association with muscle regeneration, immunohistochemical staining for utrophin, dystrophin, vimentin and desmin were performed.

MATERIALS AND METHODS

Materials

Seventeen cases with enough biopsied muscle tissue were selected out of 22 cases of DMD diagnosed at the Yongdong Severance Hospital from August, 1999 to April, 2000. DMD was diagnosed on the basis of clinicopathologic features and dystrophin gene analysis, which was performed by polymerase chain reaction (PCR). As fresh skeletal muscle tissue of normal subjects could not be obtained, 3 cases of polymyositis and 1 case of dermatomyositis were included for comparison.

Methods

A. Skeletal muscle biopsy

Skeletal muscle biopsies were obtained 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.

B. 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 at 1:50 against the 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-labeled secondary antibodies using the DAKO LSAB kit (DAKO Corporation, Carpinteria, CA, USA) at room temperature for 30 minutes. After rinsing with PBS, the sections were incubated with streptavidin peroxidase at room temperature for 30 minutes, rinsed with PBS, developed with amino-ethyl carbazole (Bimeda Corp., Foster, CA, USA), rinsed with water and finally counterstained with hematoxylin.

C. Image analysis

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

(1) Subdivision of muscle fibers by diameter

The muscle fiber diameter perpendicular to the maximum diameter was measured to overcome the distortion which occurs when a fiber is cut obliquely. The fibers were then divided into three stages according to diameter. Muscle fibers measuring less than 30 μm were defined as stage 1, those measuring 30 μm or greater but less than 50 μm as stage 2 and those measuring 50 μm or greater as stage 3.

(2) Measurement of staining intensity

The staining intensities of utrophin, vimentin and desmin were measured using a scale of 0-255. As the imaging software did not allow free-form tracing, a line was used to measure the staining intensity of utrophin and a circular field, approximating the area of an individual muscle fiber was used for vimentin and desmin.

D. Interpretation of immunohistochemistry and image analysis results

For dystrophin and utrophin, staining along the entire circumference of the sarcolemma was interpreted as correctly stained, and for vimentin and desmin, homogeneous staining of the entire cytoplasm was interpreted as correctly stained. A

cut-off value of 100 was used to define a positive stain.

E. Statistical analysis

The Chi-square test and one-way analysis of variance (ANOVA) using the SAS system were performed, and the level of significance was set at $p<0.05$.

RESULTS

Clinical findings

A. Duchenne muscular dystrophy

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

B. Inflammatory myopathy

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

Light microscopic findings

A. Duchenne muscular dystrophy (Fig. 1A)

B. Inflammatory myopathy (Fig. 1B)

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.

(2) Inflammatory myopathy

Dystrophin was positive in all 3 cases of polymyositis and in the single case of dermatomyositis (Fig. 1D).

B. Utrophin

(1) Duchenne muscular dystrophy

Utrophin was positive in 16 of 17 cases (94.0%) (Fig. 1E). 36.4% of the myofibers demonstrated positive staining. Of these, 68.7% were in stage 1, 28.9% in stage 2 and 2.5% in stage 3 (p=0.001) (Fig. 2A).

(2) Inflammatory myopathy

Utrophin was positive in 3 of 4 cases (75.0%) (Fig. 1F). 10.5% of the myofibers demonstrated positive staining. Of these, 84.6% were in stage 1 and 15.4% in stage 2 (p=0.013) (Fig. 2A).

C. Vimentin and desmin

(1) Vimentin

(A) Duchenne muscular dystrophy

Vimentin stained positively in all 15 cases stained. 34.4% of the myofibers demonstrated positive staining (Fig. 3A). Of these, 94.0% were in stage 1 and 6.0% in stage 2 (p=0.001) (Fig. 2B).

(B) Inflammatory myopathy

Vimentin stained positively in all 4 cases. 21.8% of the myofibers demonstrated positive staining (Fig. 3B). Of these, 98.6% were in stage 1 and 1.4% in stage 2 (p=0.001) (Fig. 2B).

(2) Desmin

(A) Duchenne muscular dystrophy

Desmin stained positively in all 15 cases stained. 35.4% of the myofibers demonstrated

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

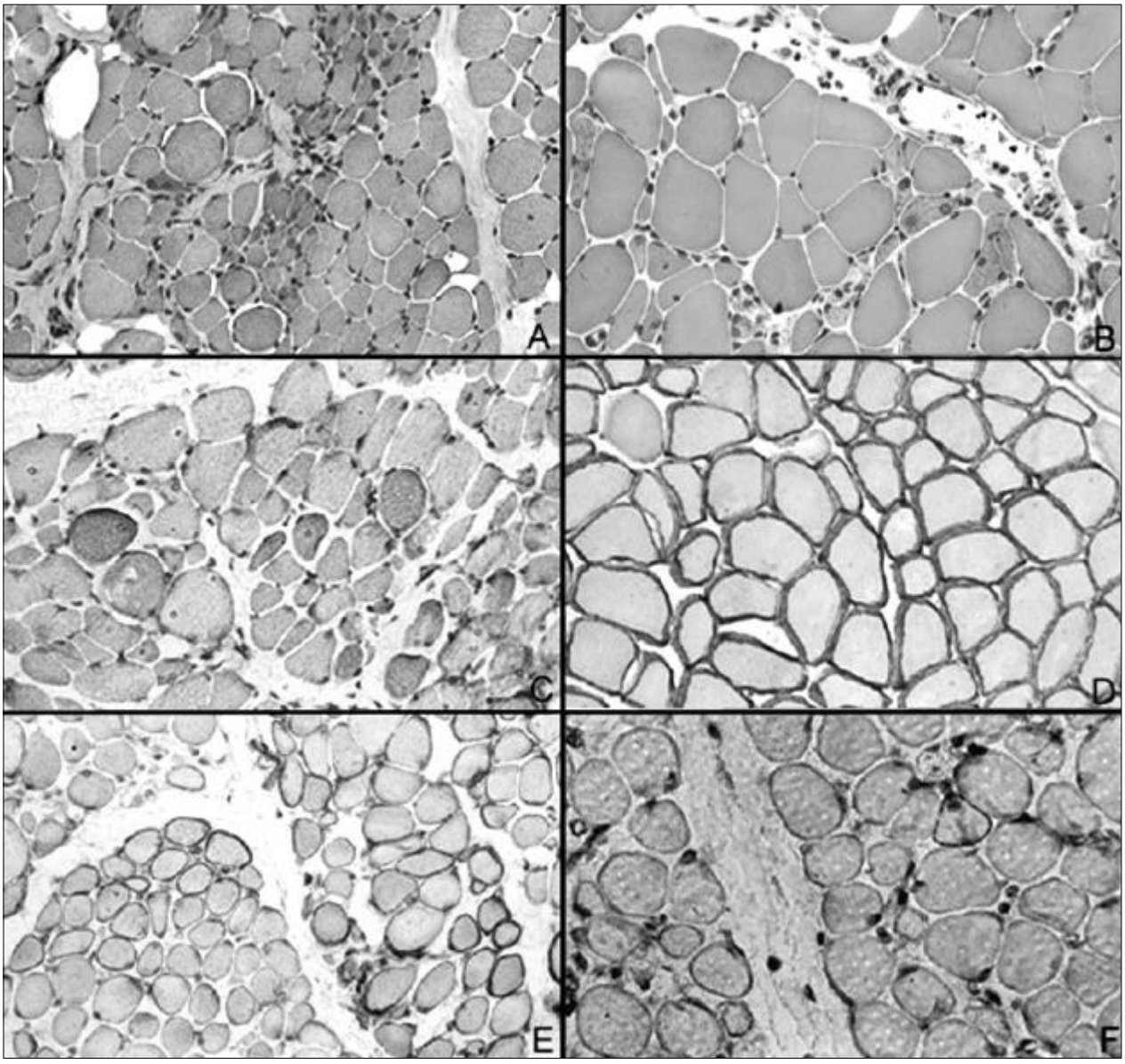

Fig. 1. Findings of hematoxylin-eosin stain (A, B) and immunohistochemical staining for dystrophin (C, D) and utrophin (E, F) in Duchenne muscular dystrophy (A, C, E) and inflammatory myopathy (B, D, F).

positive staining (Fig. 3C). Of these, 91.6% were in stage 1, 7.3% in stage 2 and 1.1% in stage 3 (p=0.001) (Fig. 2C).

(B) Inflammatory myopathy

Desmin stained positively in all 4 cases. 20.9% of the myofibers demonstrated positive staining (Fig. 3D). Of these, 95.5% were in stage 1 and 4.5% in stage 2 (p=0.001) (Fig. 2C).

Image analysis findings

A. Utrophin

(1) Duchenne muscular dystrophy

Differences in staining intensities of stages 1 and 3, and of stages 2 and 3 were statistically significant (Fig. 4A, B, and C, Table 2).

(2) Inflammatory myopathy

There was a statistically significant difference in

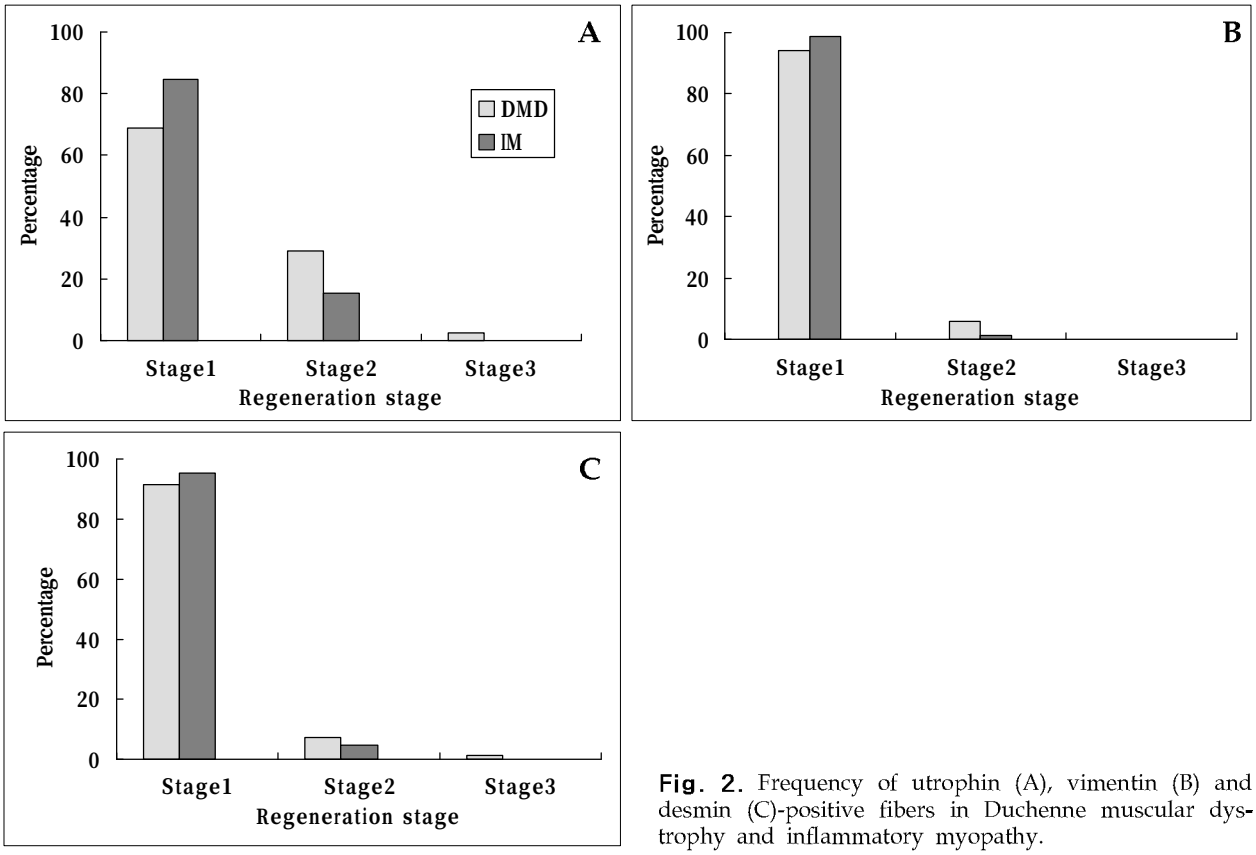

Fig. 2. Frequency of utrophin (A), vimentin (B) and desmin (C)-positive fibers in Duchenne muscular dystrophy and inflammatory myopathy.

Table 2. Staining Intensity of Utrophin, Vimentin and Desmin according to Regeneration Stage in Duchenne Muscular Dystrophy

Marker	Staining intensity(mean ± SD)		
	Stage 11	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: $< 30\,\mu$m, Stage 2: $\geq 30\,\mu$m, $< 50\,\mu$m, Stage 3; $\geq 50\,\mu$m.

staining intensity between stages 1 and 2 (Table 3).

B. Vimentin

(1) Duchenne muscular dystrophy

There was a statistically significant difference in staining intensity between the stages (Fig. 4D, E, and F, Table 2).

(2) Inflammatory myopathy

Differences in staining intensities of stages 1 and 2, and of stages 1 and 3 were statistically significant (Table 3).

C. Desmin

(1) Duchenne muscular dystrophy

There was a statistically significant difference in staining intensity between the stages (Fig. 4G, H, and I, Table 2).

(2) Inflammatory myopathy

Differences in staining intensities of stages 1 and 2, and of stages 1 and 3 were statistically

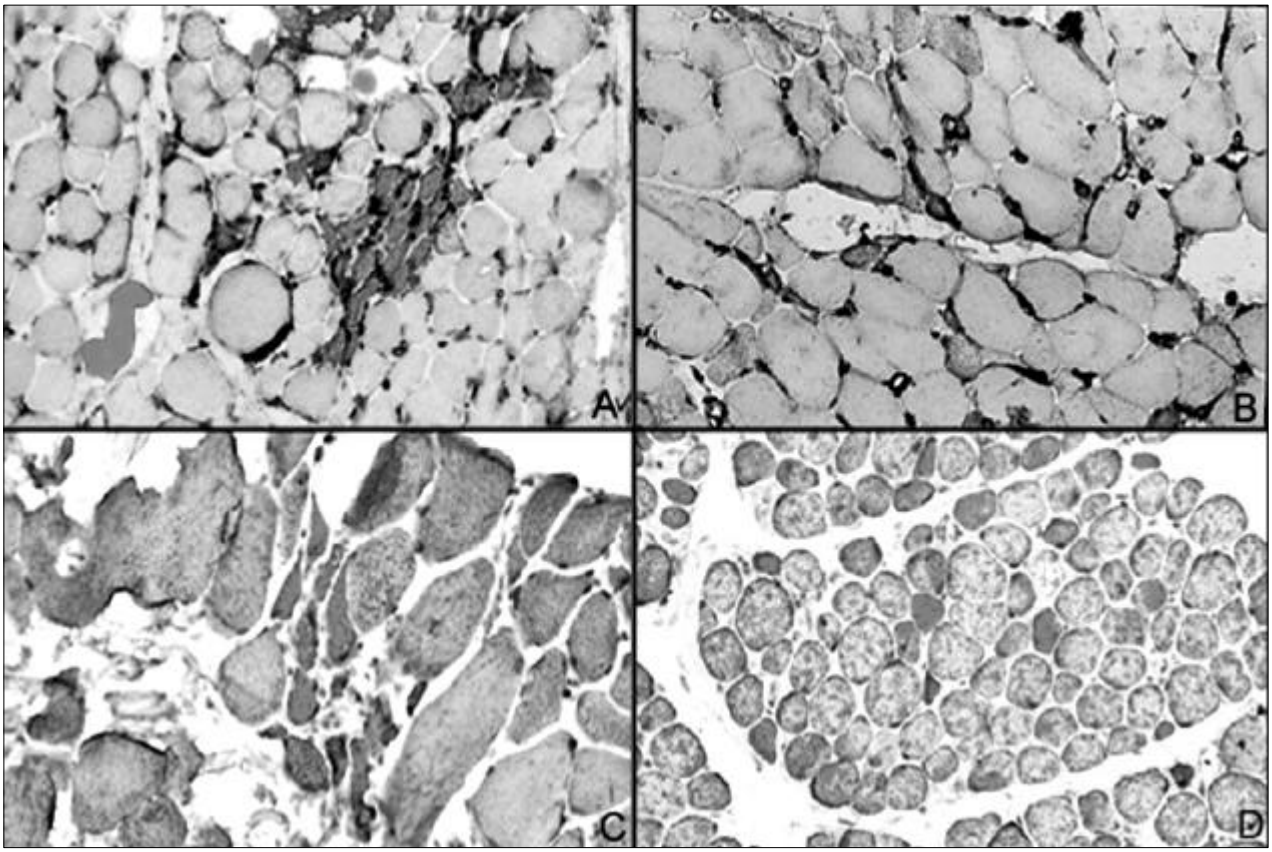

Fig. 3. Findings of immunohistochemical stains for vimentin (A, B) and desmin (C, D) in Duchenne muscular dystrophy (A, C) and inflammatory myopathy (B, D).

Table 3. Staining Intensity of Utrophin, Vimentin and Desmin according to Regeneration Stage in Inflammatory Myopathy

Marker	Staining intensity (mean ± SD)		
	Stage 11	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: $< 30\mu m$, Stage 2: $\geq 30\mu m$, $< 50\mu m$, Stage 3; $\geq 50\mu m$.

significant (Table 3).

DISCUSSION

Utrophin is a structurally homologous protein of dystrophin. Although knowledge on the distribution and regulation[23,24] of utrophin is increasing, little is known about its function. As is the case for dystrophin, the amino-terminus of utrophin binds to actin[6] and the carboxyl-terminus binds to the dystrophin-associated protein complex,[11] which raises the possibility that utrophin may play a role similar to that of dystrophin at the sarcolemma.

Recently, it has been shown in DMD that utrophin is spontaneously up-regulated anomalously at the sarcolemma, while dystrophin is

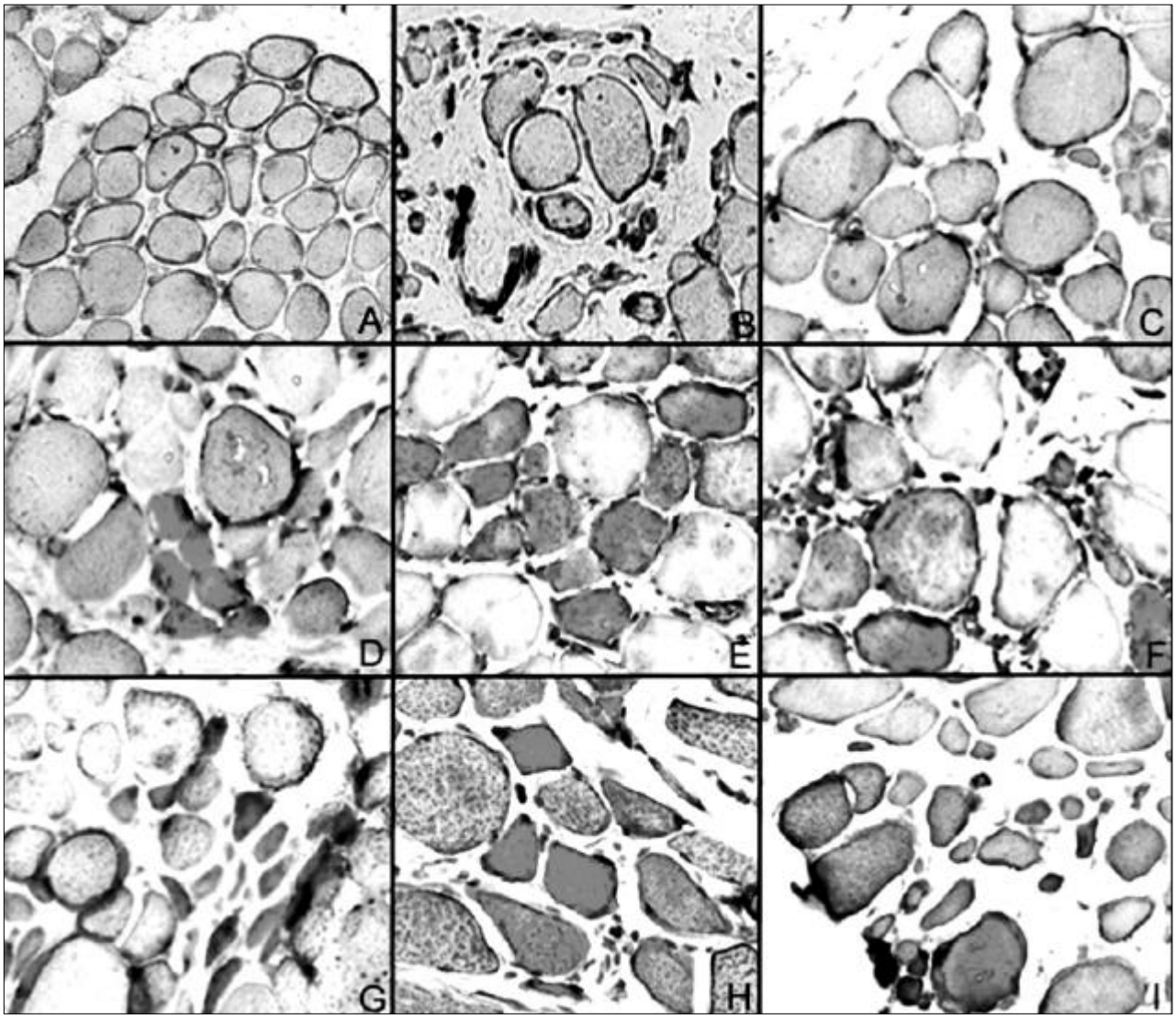

Fig. 4. Staining intensity of utrophin (A, B, C), vimentin (D, E, F) and desmin (G, H, I) in Duchenne muscular dystrophy according to regeneration stage (A, D, G: stage 1, B, E, H: stage 2, C, F, I: stage 3).

lacking. In addition, the dystrophin-associated protein level is reduced in DMD.[6-8,10-18] The mechanism of utrophin up-regulation is not yet clear.

As utrophin and dystrophin are closely related, utrophin up-regulation in DMD may be a compensatory mechanism for the dystrophin deficiency.[1,5,8,11,17,19,24] However, the up-regulated utrophin does not prevent muscle necrosis in DMD patients, and no relationship was found between the quantity and distribution of utrophin and patient age or the severity of clinical course.[26] In contrast, utrophin is reported to be up-regulated not only in DMD but also in IM, including polymyositis and dermatomyositis, and in normal skeletal muscle.[8,12,16,23] Thus, it has been argued that utrophin is expressed in regenerating fibers irrespective of the dystrophin deficiency. A recent study in hypertrophic feline muscular dystrophy demonstrated that utrophin is a regeneration-associated protein, as it was found to be transiently expressed in the early stages of regeneration and disappeared as the muscle fibers increased in size.[25]

Utrophin up-regulation may also represent a nonspecific stress reaction, like that known for

class I major histocompatibility complex protein products and heat-shock proteins, which 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.[8]

Semi-quantitative analysis of the level of expression of utrophin has been performed previously[23] as has the measurement of immunoblot intensity by image analysis,[8] but this is the first study to use image analysis to measure the staining intensities of utrophin, vimentin and desmin by immunohistochemical staining. This was performed to objectively quantify the different staining intensities in the regeneration stages of DMD. For utrophin, the staining intensity was higher in the small regenerating fibers in both DMD and IM. For both vimentin and desmin, a statistically significant difference was observed in the staining intensities of the three DMD stages. In IM, the staining intensity was higher in the small regenerating fibers. One of the factors responsible for the variability in the results is that the frequency of muscle fibers in each stage was unevenly distributed. A shortcoming in the measurement of staining intensity by immunohistochemical staining is that there was a slight variation in the staining quality between cases. Also, as the image analysis software system did not allow free-form tracing, we could not improve the accuracy of the staining intensity measurement.

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

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