

## Reduced expression of sarcospan in muscles of Fukuyama congenital muscular dystrophy

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**Summary.** Expression profiles of sarcospan in muscles with muscular dystrophies are scarcely reported. To examine this, we studied five Fukuyama congenital muscular dystrophy (FCMD) muscles, five Duchenne muscular dystrophy (DMD) muscles, five disease control and five normal control muscles. Immunoblot showed reactions of sarcospan markedly decreased in FCMD and DMD muscle extracts. Immunohistochemistry of FCMD muscles showed that most large diameter myofibers expressed sarcospan discontinuously at their surface membranes. Immature small diameter FCMD myofibers usually did not express sarcospan. Immunoreactivity of sarcospan in DMD muscles was similarly reduced. With regard to dystroglycans and sarcoglycans, immunohistochemistry of FCMD muscles showed selective deficiency of glycosylated  $\alpha$ -dystroglycan, together with reduced expression of  $\beta$ -dystroglycan and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -sarcoglycans. Although the expression of glycosylated  $\alpha$ -dystroglycan was lost, scattered FCMD myofibers showed positive immunoreaction with an antibody against the core protein of  $\alpha$ -dystroglycan. The group mean ratios of sarcospan mRNA copy number versus GAPDH mRNA copy number by real-time RT-PCR showed that the ratios between FCMD and normal control groups were not significantly different ( $P > 0.1$  by the two-tailed t test). This study implied either O-linked glycosylation defects of  $\alpha$ -dystroglycan in the Golgi apparatus of FCMD muscles may lead to decreased expression of sarcoglycan and sarcospan molecules, or selective deficiency of glycosylated  $\alpha$ -dystroglycan due to impaired glycosylation in FCMD muscles may affect the molecular integrity of the basal lamina of myofibers.

This, in turn, leads to decreased expression of sarcoglycans, and finally of sarcospan at the FCMD myofiber surfaces.

**Key words:** Sarcospan, Muscular dystrophy, Immunoblot, Real-time RT-PCR, Immunofluorescence

### Introduction

Sarcospan is a 25-kDa dystrophin-associated protein of the dystrophin-glycoprotein complex (DGC) (Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992; Crosbie et al., 1997). Constituent proteins of the DGC are structurally organized into three distinct subcomplexes, which include cytoskeletal proteins (dystrophin, dystrobrevins and syntrophins), dystroglycans ( $\alpha$ - and  $\beta$ -subunits), sarcoglycans ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$  and  $\epsilon$ -subunits) and sarcospan.  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -sarcoglycans associate to form a heterotetrameric complex (Araishi et al., 1999). The sarcoglycan complex in vascular and visceral smooth muscle consists of  $\epsilon$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -sarcoglycans, and is associated with sarcospan (Barresi et al., 2000). Sarcospan has been cloned and sequenced by Crosbie et al. (1997), but the functional role of sarcospan is unknown. This study investigated the expression of sarcospan and associated proteins, such as sarcoglycans and dystroglycans, to speculate on the functional role of sarcospan.  $\beta$ -Dystroglycan and sarcoglycans have one transmembrane domain in each molecule (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992; Crosbie et al., 1997) and sarcospan is predicted to contain four transmembrane domains (Crosbie et al., 1997). These characteristics of sarcospan make it unique among other members of DGC. The predicted topology of sarcospan with four transmembrane domains and a large extracellular loop is similar to that of the tetraspan

superfamily of proteins (Wright and Tomlinson, 1994; Crosbie et al., 1997). Although the function of the tetraspan superfamily members is not clear, many of them are associated with integrins (Maecker et al., 1997) and have been implicated in the regulation of cell development, proliferation, activation, motility (Wright and Tomlinson, 1994) and in the process of malignancy (Heighway et al., 1996). Crosbie et al. (1999) described that sarcospan is enriched at the myotendinous junction (a focal adhesion-like structure) and neuromuscular junction. From these observations, one plausible speculation is that sarcospan seems to act as a force transmitting molecule. However, the skeletal muscle pathology of sarcospan knockout mouse shows no gross abnormalities (Lebakken et al., 2000). Furthermore, Peter et al. (2007) reported that the disrupted mechanical stability of DGC causes severe muscular dystrophy in sarcospan overexpressing transgenic mice. We thought that examination of the expression of the sarcoglycan-sarcospan complex, especially that of sarcospan, in muscular dystrophy with severe muscle weakness, such as Fukuyama congenital muscular dystrophy (FCMD), may provide suggestions about the functional role(s) of sarcospan. We also studied the sarcospan expression of biopsied diseased muscles of different genetic or molecular causes, such as Duchenne muscular dystrophy (DMD). We used the expression of  $\beta$ -spectrin as the marker of the myofiber plasma membrane, because plasma membranes of degenerated myofibers frequently disappear partially or totally. Thus, we investigated the expression of sarcospan in relation to other transmembrane glycoprotein members of DGC in the FCMD muscles.

## Materials and Methods

### *Muscle samples*

All muscle samples were biopsied from quadriceps femoris muscles under local anesthesia. Muscle samples contained five FCMD muscles, five DMD muscles, three muscles with myotonic dystrophy and two muscles with facioscapulohumeral muscular dystrophy (FSHD). Children (4 boys and 1 girl aged 7 months to 1 year) affected with FCMD had muscle weakness and hypotonia from infancy, delayed motor milestone, myopathic faces, mental retardation, high serum creatine kinase (CK) values and myopathic changes shown by electromyograms (Fukuyama et al., 1981). Among the five child patients, two patients were confirmed to have FCMD by genetic analysis. The diagnosis of FCMD in the remaining three child patients was made by typical clinical signs and laboratory examinations, such as increased serum CK values and myopathic electromyograms. Boys aged 1 month to 8 years affected with DMD had proximal muscle weakness, atrophy in various degrees, calf pseudohypertrophy and markedly high serum CK levels. The diagnosis of DMD was confirmed by examination of leukocyte genomic DNA

and negative dystrophin immunostaining of biopsied muscles. Myotonic dystrophy and FSHD were diagnosed by their characteristic clinical signs. Muscle samples of myotonic dystrophy and FSHD patients (4 males and 1 female aged 23 to 45 years) served as the disease control. For normal control specimens, five histochemically normal biopsy specimens of quadriceps femoris muscles were collected from 5 patients (2 males and 3 females aged 10 to 53 years) who were thought to have myopathy, but were free of neuromuscular disorders after histochemical and immunologic examinations. All muscle samples were taken under informed consent. The procedure of muscle biopsy was approved by the ethics committee of Showa University.

### *Immunoblot analysis*

Immunoblot analyses of sarcospan,  $\beta$ -spectrin and dystrophin in the histochemically normal quadriceps femoris muscles and those of children with FCMD and DMD were done by using previously described methods (Wakayama et al., 1995) with minor modifications. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was done for sarcospan (Table 1) on a 12.5% homogeneous gel, and for  $\beta$ -spectrin (Table 1) and dystrophin (Dys3) (Table 1) on a 3-10% gradient gel. The protein was transferred from the gel to a clear blot P membrane sheet (ATTO, Tokyo, Japan) using horizontal electrophoresis at 108mA for 90 min. at room temperature. Immunostainings were done with rabbit anti-sarcospan antibody diluted to 5 $\mu$ g IgG/ml, rabbit anti- $\beta$ -spectrin antiserum diluted to 1:200 and mouse monoclonal anti-dystrophin antibody diluted to 1:500 (Table 1).

### *Immunohistochemistry*

Antibodies against sarcospan,  $\beta$ -dystroglycan and a skeletal muscle specific isoform of  $\beta$ -spectrin were generated in our laboratory (Table 1). The muscle biopsy specimens were immediately frozen in isopentane cooled with liquid nitrogen. Frozen 6  $\mu$ m-thick cross-sections of the muscles were placed on cover slips and were incubated with primary rabbit anti-sarcospan antibody (Table 1) diluted to 5  $\mu$ g IgG/ml. Serial sections of muscles were incubated with primary rabbit anti- $\beta$ -spectrin antiserum (Table 1) diluted to 1:200. Muscle sections from the five FCMD children were immunostained with mouse monoclonal anti-neonatal myosin antibody (Table 1) diluted to 1:50. The immunohistochemistry of FCMD and normal control muscles was done using anti  $\alpha$ - (IIH6C4 and a core protein) and  $\beta$ -dystroglycan, merosin, dystrophin,  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -sarcoglycans antibodies. Two antibodies of  $\alpha$ -dystroglycan were used: IIH6C4 and a goat polyclonal antibody against  $\alpha$ -dystroglycan core protein raised against a mixture of recombinant  $\alpha$ -dystroglycan N-terminal and C-terminal domains expressed in *Escherichia coli*, and was affinity-purified using a

## Sarcospan in Fukuyama muscular dystrophy

recombinant  $\alpha$ -dystroglycan from HEK 293 cells (Table 1). Table 1 lists the dilution titer of the respective antibody. The muscle sections were washed and were incubated with fluorescein-isothiocyanate-conjugated secondary rabbit anti-goat, swine anti-rabbit or rabbit anti-mouse antibody diluted 1:50 overnight at 4°C. The muscle samples were examined by fluorescence microscopy.

### Analysis of immunohistochemically stained muscles

Muscle specimens with immunohistochemical staining by anti- $\beta$ -spectrin and anti-sarcospan antibodies were examined, and photographs were taken and printed at a final magnification of x250 as described previously (Wakayama et al., 2006). The immunostaining patterns of the surface membrane of myofibers were classified into three patterns: group 1, continuously positive immunostaining pattern with more than 90% myofiber surface immunolabeling; group 2, partially positive immunolabeling pattern with 10 to 90% myofiber surface immunolabeling; group 3, negative immunostaining pattern with less than 10% myofiber surface immunolabeling. The immunostaining patterns of each muscle were analyzed using about 200 myofibers in each case. The group mean percentage of each immunostaining pattern was calculated for FCMD, DMD, disease control and normal control groups. The

difference in the group mean percentage for each immunostaining pattern in each muscle group was statistically compared by using the two-tailed t test. FCMD muscle sections immunostained with anti-neonatal myosin antibody were evaluated qualitatively.

### Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) for human sarcospan

Total RNA was extracted from approximately 30 mg of each FCMD or normal control muscle samples by an acid-phenol extraction reagent (TRIzol, Code 15596-026; Gibco BRL, Rockville, MD, USA). The concentration of sarcospan mRNA was estimated by using real-time RT-PCR. The oligonucleotide primers were designed from the human sarcospan sequence (GenBank AF016028): sense strand (sarcospanF) 5'-CGCAGCTCACACAGTTTACC-3' and antisense strand (sarcospanR) 5'-GACACCCACATAGAAGACC-3' in the sarcospan gene at exon 3. GAPDH mRNA was amplified as the internal gene control to explain variations in RNA levels between different samples. The oligonucleotide primers of GAPDH were: sense strand (GAPDHF) 5'-ACCACAGTCCATGCCATCAC-3'; antisense strand (GAPDHR) 5'-TCCACCACCCTGTTGCTGTA-3'. To generate the standard curves of human sarcospan and GAPDH, we synthesized primers by adding the T7 promoter sequence to the forward

**Table 1.** Primary antibodies.

Antigen	Antibody	Concentration of dilution for IHC	Reference or source
$\alpha$ -Dystroglycan, rabbit skeletal muscle membrane preparation, Clone IH6C4	Mouse monoclonal (IgM)	1:50	Upstate Temecula CA, USA
$\alpha$ -Dystroglycan core protein, mixture of recombinant $\alpha$ -dystroglycan N- and C-terminal domains	Goat polyclonal (IgG)	5 $\mu$ g of IgG/ml	Kanagawa et al. (unpublished data)
$\beta$ -Dystroglycan, amino acid residues 880-894 (C-terminus), synthetic peptide	Sheep polyclonal	5 $\mu$ g of IgG/ml	Ibraghimov-Beskrovnya et al., 1992; Wakayama et al., 1995
Dystrophin (Dys3), amino terminal domain (between amino acids 321-494) of human dystrophin	Mouse monoclonal (IgG)	1:20	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
Merosin, purified human merosin	Mouse monoclonal (IgG)	1:30	Chemicon International Inc., Temecula CA, USA
Neonatal myosin, rabbit neonatal myosin heavy chain	Mouse monoclonal (IgG)	1:50	Vector Laboratories Inc., CA, USA
$\alpha$ -Sarcoglycan, amino acid residues 217-289 of the rabbit $\alpha$ -sarcoglycan sequence, fusion protein	Mouse monoclonal (IgG)	1:100	Ylem, Rome, Italy
$\beta$ -Sarcoglycan, fusion protein RBSG-NT of the human $\beta$ -sarcoglycan sequence, fusion protein	Mouse monoclonal (IgG)	1:50	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
$\gamma$ -Sarcoglycan, amino acid residues 167-178 of the rabbit $\gamma$ -sarcoglycan sequence, synthetic peptide	Mouse monoclonal (IgG)	1:100	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
$\delta$ -Sarcoglycan, amino acid residues 1-19 at the N-terminus of the human $\delta$ -sarcoglycan sequence, synthetic peptide	Mouse monoclonal (IgG)	1:50	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
Sarcospan CAADRQPRGQQRQGDAAAGPD N-terminal domain of human sarcospan, synthetic peptide	Rabbit polyclonal	5 $\mu$ g of IgG/ml	Crosbie et al., 1997; Hayashi et al., 2006
$\beta$ -Spectrin (C-terminus of 270-kDa muscle isoform), synthetic peptide	Rabbit polyclonal	1:200	Porter et al., 1992; Wakayama et al., 1995

IHC: Immunohistochemistry.

## Sarcospan in Fukuyama muscular dystrophy

primer and the oligo-dT sequence to the reverse primer for sarcospan and GAPDH genes. The total RNA (100 ng) extracted from normal human skeletal muscle was reverse transcribed and amplified by PCR using a One Step RNA PCR Kit (TaKaRa Bio Inc., Shiga, Japan, Code RR024A). After 2% agarose gel electrophoresis of the RT-PCR products, bands corresponding to human sarcospan and GAPDH were extracted, purified and sequenced to confirm their gene sequences. By using their double strand DNA as templates, single strand RNA was made by using an In Vitro Transcription T7 Kit (TaKaRa Bio Inc., Shiga, Japan, Code 6140) and the copy number was calculated for human sarcospan and GAPDH products. Samples of serial dilution were prepared for human sarcospan and GAPDH mRNAs. To generate the standard curve for human sarcospan mRNA, the diluted samples were reverse transcribed for 10 min at 50°C followed by 30 cycles of PCR (5 sec and 10 sec at 94°C and 50°C, respectively, and 20 sec at 72°C) with the reaction mixture containing 1xPCR buffer, 0.4μM each of primer pairs, 5mM magnesium chloride, 0.8U/μl ribonuclease inhibitor, 0.1U/μl reverse transcriptase, 0.1U/μl Taq polymerase and 1mM dNTP mixture using Real Time One Step RNA PCR Kit (TaKaRa Bio Inc., Shiga, Japan, Code RR026A). To calculate the copy numbers of human sarcospan mRNAs of the biopsied muscles, the extracted total RNA samples (50 ng) were reverse transcribed and the resulting cDNA was amplified by PCR similarly. To generate the standard curve for GAPDH mRNA and to calculate the copy numbers of GAPDH mRNA of the biopsied muscles, the samples (50ng) were reverse

transcribed for 10 min at 50°C, followed by 30 cycles of PCR (30 sec and 20 sec at 94°C and 62°C, respectively, and 30 sec at 72°C) in the same reaction mixture as described for sarcospan mRNA. From the standard curve, the copy numbers of mRNA of GAPDH were calculated, and finally the ratio of human sarcospan mRNA copy number versus GAPDH mRNA copy number were calculated in each muscle. Fifteen biopsied muscles were analyzed in duplicate by using this method and the mean was used in each case. The statistical difference between the FCMD, DMD and normal control groups was evaluated using the two-tailed t test.

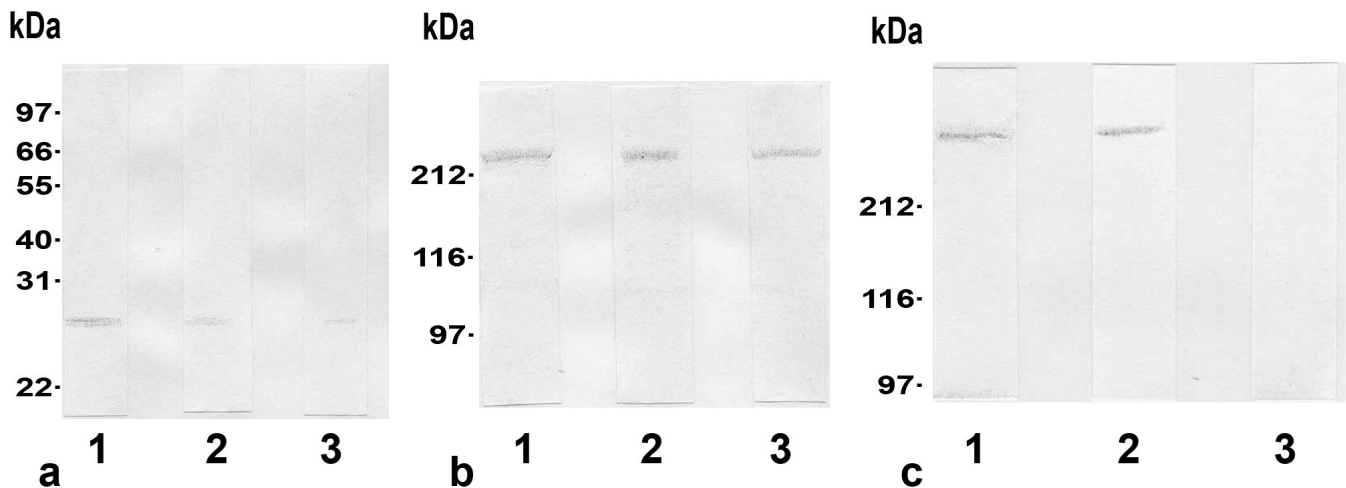
## Results

### Immunoblot analysis

Immunoblot analysis showed that the anti-sarcospan antibody reacted with 25-kDa protein extracts of normal muscles, but the reactions of sarcospan markedly decreased in FCMD and DMD muscle extracts (Fig. 1a). The reactions of anti-β-spectrin (Fig. 1b) antibody in FCMD and DMD muscle extracts were similar to those in extracts of normal control muscles. The reactions of anti-dystrophin (Fig. 1c) antibody were negative in DMD, but those in FCMD muscle extracts were similar to those in normal muscle extracts.

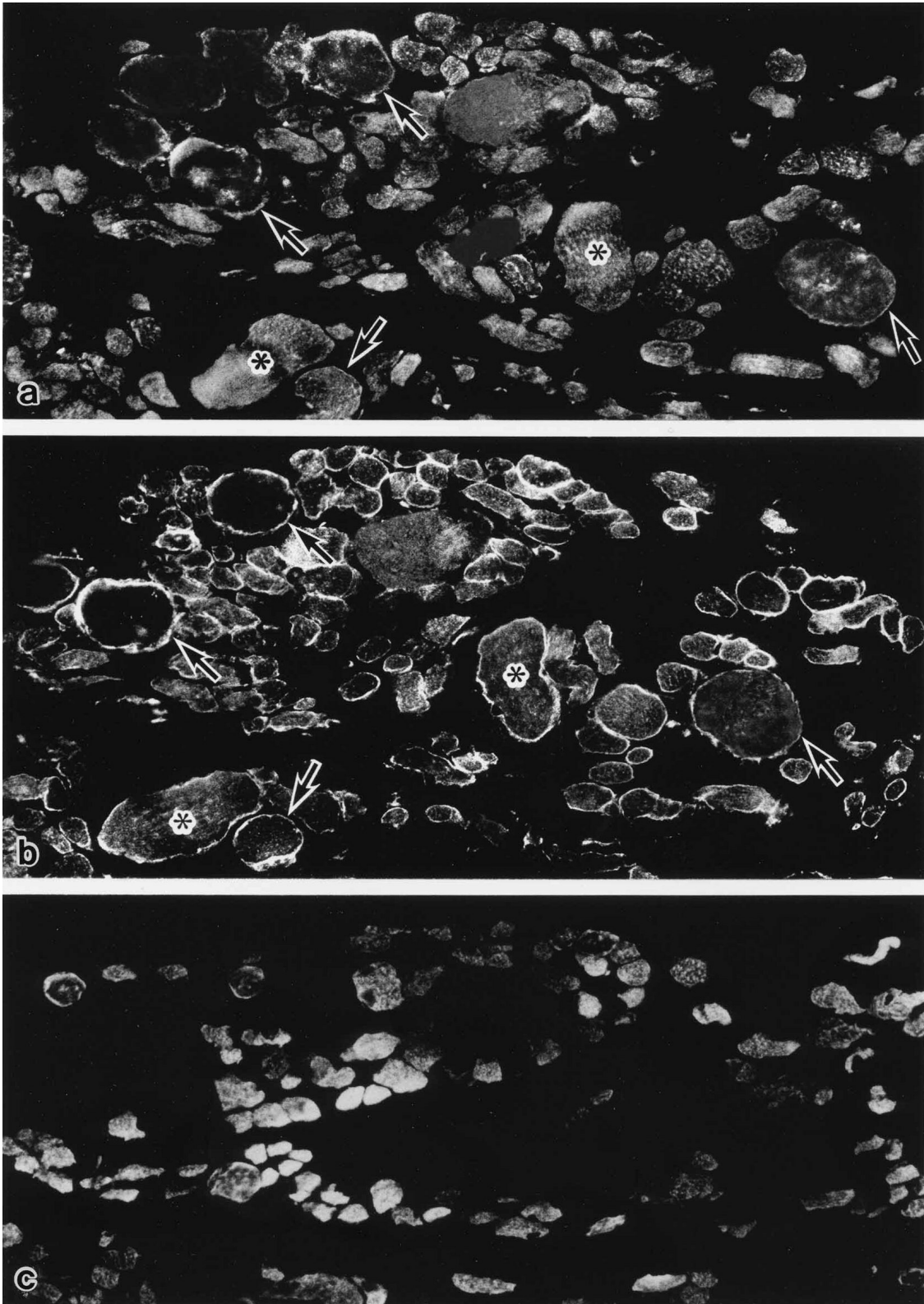
### Immunohistochemistry

FCMD muscles showed that most large diameter myofibers expressed sarcospan discontinuously at their



**Fig. 1.** Immunoblots of sarcospan (a), β-spectrin (b) and dystrophin (c) expressions in normal human quadriceps femoris muscles (lane 1 of a, b and c, respectively), in muscles of children with Fukuyama congenital muscular dystrophy (FCMD) (lane 2 of a, b and c, respectively) and in muscles of boys with Duchenne muscular dystrophy (DMD) (lane 3 of a, b and c, respectively). Electrophoresis and blotting were done as described in Materials and Methods. In normal human muscle extracts, bands of sarcospan, β-spectrin and dystrophin were at 25kDa, 270kDa and 427kDa, respectively (lane 1 of a, b and c, respectively). The reaction for sarcospan both in FCMD and DMD muscle extracts markedly decreased compared with that of normal muscle extracts (a); while the reaction for β-spectrin in FCMD and DMD muscle extracts showed almost normal intensity to a slight decrease compared with normal control muscle extracts (b). Reactions for dystrophin in extracts of normal and FCMD muscles had almost the same intensity, whereas the reaction for dystrophin in extracts of DMD muscles showed no band (c). Numbers to the left of each figure are the molecular masses of standards.

*Sarcospan in Fukuyama muscular dystrophy*



**Fig. 2.** Immunohistochemical staining of serial muscle sections of FCMD muscles with anti-sarcospan (**a**), anti- $\beta$ -spectrin (**b**) and anti-neonatal myosin (**c**) antibodies. Sporadic large diameter FCMD myofibers (arrows in **a**) express the sarcospan discontinuously at their myofiber surface, although large diameter FCMD myofibers (asterisks in **a**) do not necessarily express the sarcospan molecule. Most small diameter FCMD myofibers do not express sarcospan at their surface membrane (**a**). In **b**, both small and large diameter FCMD myofibers, including large diameter fibers with arrows and asterisks, express  $\beta$ -spectrin more or less at their myofiber surface. In **c**, small diameter FCMD myofibers contain immature myosin with neonatal myosin positive immunoreactivity, while large diameter FCMD myofibers, including those with arrows and asterisks in **a** and **b**, show negative immunoreactivity with anti-neonatal myosin antibody.  $\times 230$

surface membranes (Fig. 2a), irrespective of the presence of  $\beta$ -spectrin in most FCMD myofibers (Fig. 2b). Small diameter FCMD myofibers usually did not express sarcospan at their surface membranes (Fig. 2a); these small diameter myofibers contained myosin with positive anti-neonatal myosin antibody reactivity (Fig. 2c). However, normal human skeletal myofibers, stained with anti-sarcospan (Fig. 3a) and anti- $\beta$ -spectrin (Fig. 3b) antibodies, showed immunoreaction at their myofiber surfaces. In DMD muscles, most myofibers were immunonegative for sarcospan (Fig. 3c), although most of these myofibers were immunostained with anti- $\beta$ -spectrin antibody in serial muscle section (Fig. 3d), irrespective of the absence of dystrophin (Fig. 3e). Interestingly, a small, but substantial, number of DMD myofibers showed partial sarcospan immunoreactivity (Fig. 3c), and again the surface labeling of most of these DMD myofibers with anti-sarcospan antibody was less than myofibers immunostained with anti- $\beta$ -spectrin antibody in serial muscle section (Fig. 3d). Myofibers of disease control muscles, including myotonic dystrophy and FSHD muscles, showed apparently continuous expression of sarcospan at their myofiber surface (Fig. 3f,g), as also seen in the normal control myofiber surface (Fig. 3a). The immunohistochemical studies of FCMD muscles using anti- $\alpha$ -,  $\beta$ -dystroglycan, merosin, dystrophin and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -sarcoglycan antibodies showed that glycosylated  $\alpha$ -dystroglycan expression was selectively lost in skeletal muscles of FCMD children, although the expression of other molecules was detected in many fibers (Fig. 4a-p). The co-labeling studies of serial sections of FCMD muscles revealed that reduced expression was more evident in the sarcospan molecule than in  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -sarcoglycan molecules (Fig. 5a-h). Clear and continuous surface immunolabeling of FCMD myofibers with anti-sarcoglycan antibody also showed positive immunoreaction with anti-sarcospan antibody; while FCMD myofibers with reduced expression of sarcoglycan revealed negative expression of sarcospan (Fig. 5a-h). Sarcoglycan immunonegative myofibers tended to belong to small diameter FCMD myofibers that were most likely to show sarcospan immunonegative reaction. The  $\alpha$ -dystroglycan expression using an antibody directed against the core protein showed that antibody immunoreactivity of scattered FCMD large diameter myofibers was positive (Fig. 6a), but the sarcospan expression of these myofibers was reduced (Fig. 6b) and the expression of glycosylated  $\alpha$ -dystroglycan in these myofibers appeared to be lost (Fig. 6c). Negatively immunoreactive FCMD myofibers with an antibody directed against the core protein of  $\alpha$ -dystroglycan (Fig. 6a) tended to be small diameter myofibers that were immunopositive with an antibody against neonatal myosin (Fig. 6d). Expression of glycosylated  $\alpha$ -dystroglycan in myofibers with myotonic dystrophy appeared to be normal except for occasional small diameter myofibers in which the immunoreactivity appeared to be slightly faint and the immunoreactivity of an antibody against the neonatal myosin was positive.

#### Semiquantitative analysis of sarcospan immunoreactivity of FCMD and DMD muscles

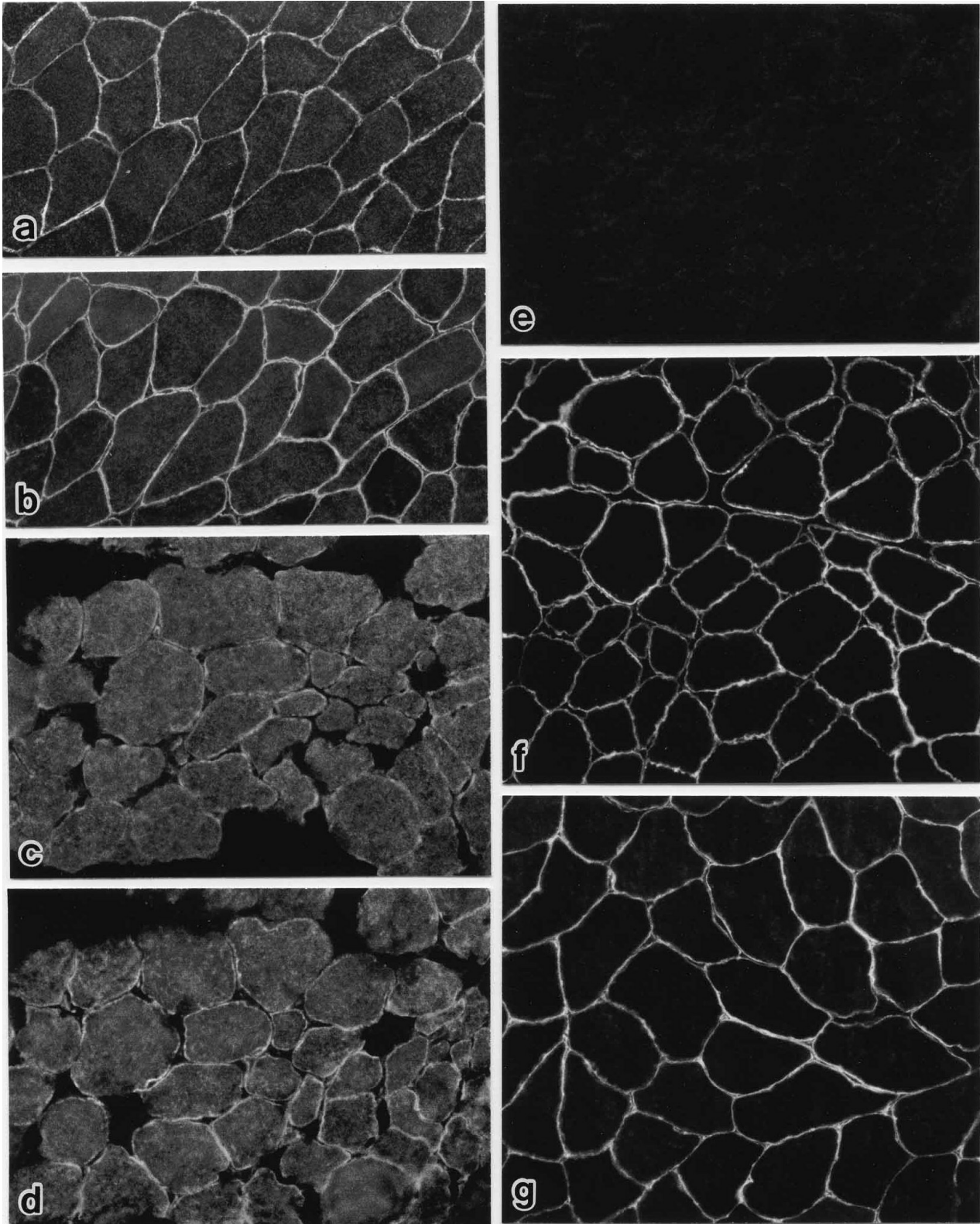
From the classification of immunostaining patterns into groups 1 to 3, the group mean percentages of groups 1, 2 and 3 FCMD myofibers stained with anti-sarcospan antibody were 2.0%, 25.3% and 72.7%, respectively, and those of groups 1, 2 and 3 FCMD myofibers stained with anti- $\beta$ -spectrin antibody were 46.2%, 38.7% and 15.1%, respectively (Table 2). DMD muscles immunostained with anti-sarcospan and anti- $\beta$ -spectrin antibodies showed immunostaining patterns similar to those of FCMD muscles, irrespective of the absence of dystrophin (Fig. 3c-e). From the classification of groups 1 to 3, the group mean percentages of groups 1, 2 and 3 DMD myofibers stained with anti-sarcospan antibody were 0.9%, 21.5% and 77.6%, respectively, and those of groups 1, 2 and 3 DMD myofibers stained with anti- $\beta$ -spectrin antibody were 45.6%, 43.4% and 11.0%, respectively (Table 2). The disease control muscle samples immunostained with anti-sarcospan and anti- $\beta$ -spectrin antibodies contained fewer myofibers with the group 2 immunostaining pattern (Table 2). The group mean percentages of sarcospan- and  $\beta$ -spectrin-immunonegative myofibers were 72.7% and 15.1%, respectively, in the FCMD group, and were 77.6% and 11.0%, respectively, in the DMD group. The group mean percentages of sarcospan-immunonegative myofibers in FCMD and DMD groups were significantly more numerous than  $\beta$ -spectrin-immunonegative myofibers

**Table 2.** Immunoreaction for  $\beta$ -spectrin and sarcospan in dystrophic muscles.

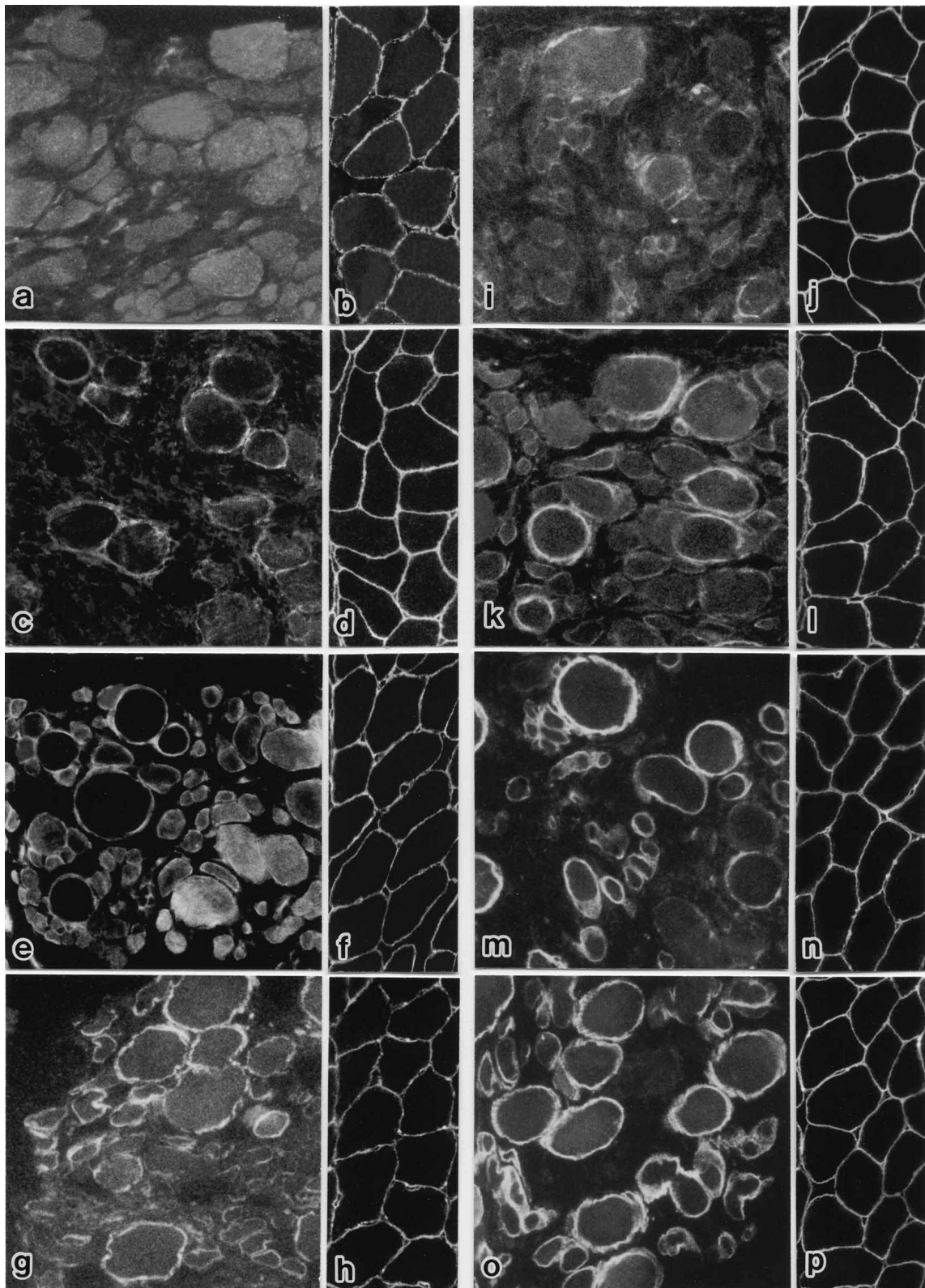
cases	anti- $\beta$ -spectrin immunoreactivity*		
	(1)	(2)	(3)
5 FCMD	46.2 $\pm$ 10.8	38.7 $\pm$ 6.0	15.1 $\pm$ 5.1
5 DMD	45.6 $\pm$ 18.8	43.4 $\pm$ 6.7	11.0 $\pm$ 2.2
5 DC	88.3 $\pm$ 2.6	11.7 $\pm$ 2.6	0
5 NC	95.2 $\pm$ 1.1	4.8 $\pm$ 1.1	0
cases	anti-sarcospan immunoreactivity*		
	(1)	(2)	(3)
5 FCMD	2.0 $\pm$ 0.9	25.3 $\pm$ 3.6	72.7 $\pm$ 4.3
5 DMD	0.9 $\pm$ 0.5	21.5 $\pm$ 3.4	77.6 $\pm$ 3.6
5 DC	88.4 $\pm$ 3.1	11.6 $\pm$ 3.1	0
5 NC	94.6 $\pm$ 1.2	5.4 $\pm$ 1.2	0

\*: group mean percentage of myofibers  $\pm$  standard error of the mean. (1)=group 1: continuously positive immunostaining pattern with more than 90% myofiber surface immunolabeling and normal immunoreactivity. (2)=group 2: partially positive immunostaining pattern with 10 to 90% myofiber surface immunolabeling and normal immunoreactivity. (3)=group 3: negative immunostaining pattern with less than 10% myofiber surface immunolabeling. DMD: Duchenne muscular dystrophy; FCMD: Fukuyama congenital muscular dystrophy; DC: disease control (myotonic dystrophy + facioscapulohumeral dystrophy); NC: normal control.

*Sarcospan in Fukuyama muscular dystrophy*



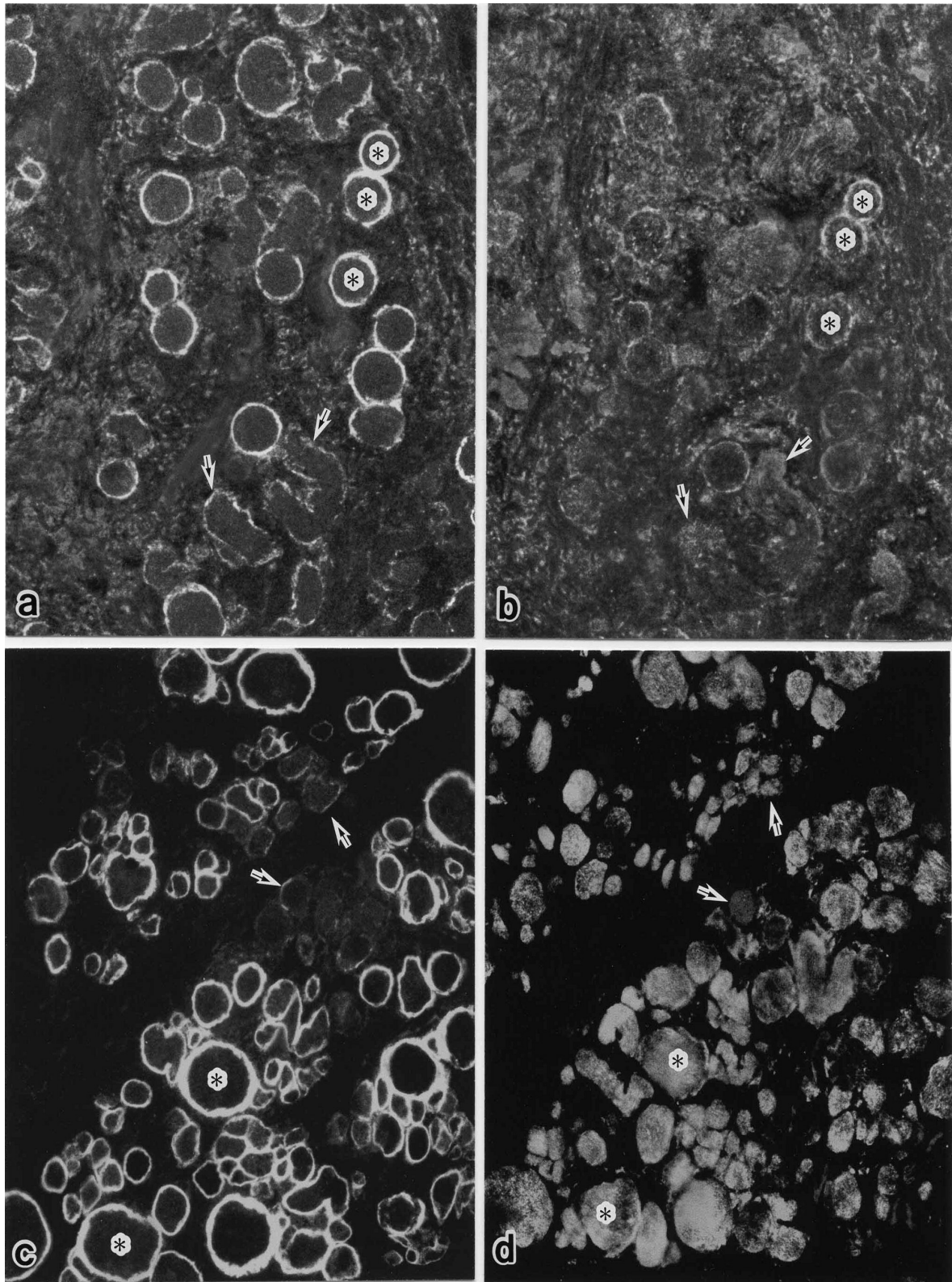
**Fig. 3.** Immunohistochemical staining of normal control muscle with anti-sarcospan (**a**) and anti- $\beta$ -spectrin (**b**) antibodies, that of DMD muscle with anti-sarcospan (**c**), anti- $\beta$ -spectrin (**d**) and anti-dystrophin (**e**) antibodies and that of myotonic dystrophy muscle (**f**) and facioscapulohumeral muscular dystrophy (FSHD) muscle (**g**) with anti-sarcospan antibody. Immunoreactivity is noted continuously at the normal control myofiber surface with anti-sarcospan (**a**) and anti- $\beta$ -spectrin (**b**) antibodies. In DMD muscle, immunoreactivity with anti-sarcospan antibody (**c**) is markedly reduced compared with that of anti- $\beta$ -spectrin antibody (**d**) and anti-dystrophin antibody immunoreactivity is negative (**e**). Anti-sarcospan antibody immunoreactivity is nearly normal in myotonic dystrophy (**f**) and FSHD (**g**) muscles. x 270

*Sarcospan in Fukuyama muscular dystrophy*

**Fig. 4.** Immunohistochemical staining of FCMD and normal control muscles with anti-glycosylated  $\alpha$ -dystroglycan (IIH6C4) (**a, b**), anti- $\beta$ -dystroglycan (**c, d**), anti-merosin (**e, f**), anti- $\alpha$ -sarcoglycan (**g, h**), anti- $\beta$ -sarcoglycan (**i, j**), anti- $\gamma$ -sarcoglycan (**k, l**), anti- $\delta$ -sarcoglycan (**m, n**) and anti-dystrophin (**o, p**) antibodies. Glycosylated  $\alpha$ -dystroglycan expression is absent, while expression of  $\beta$ -dystroglycan,  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -sarcoglycan is more or less reduced, and that of merosin and dystrophin is nearly normal compared with that of normal control muscles immunostained with antibodies raised against the respective molecule. x 240

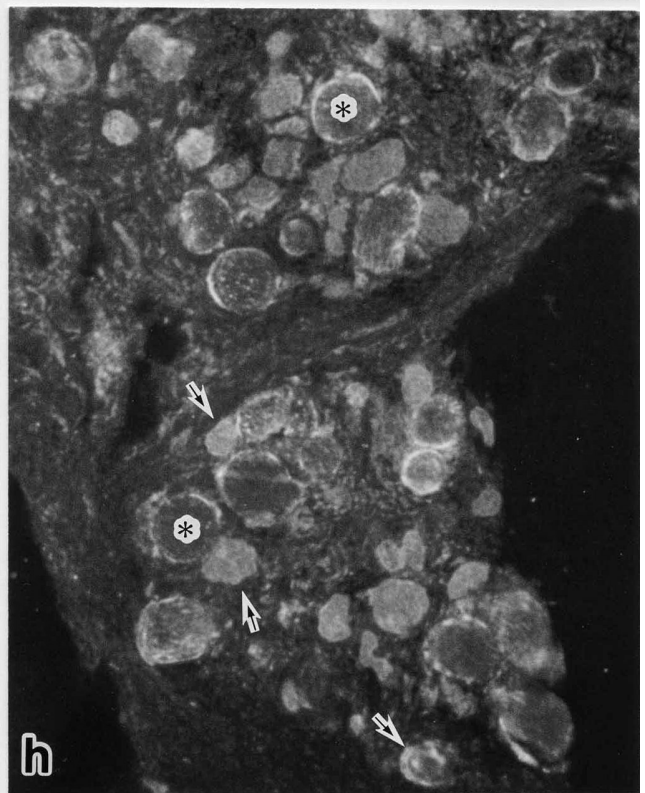
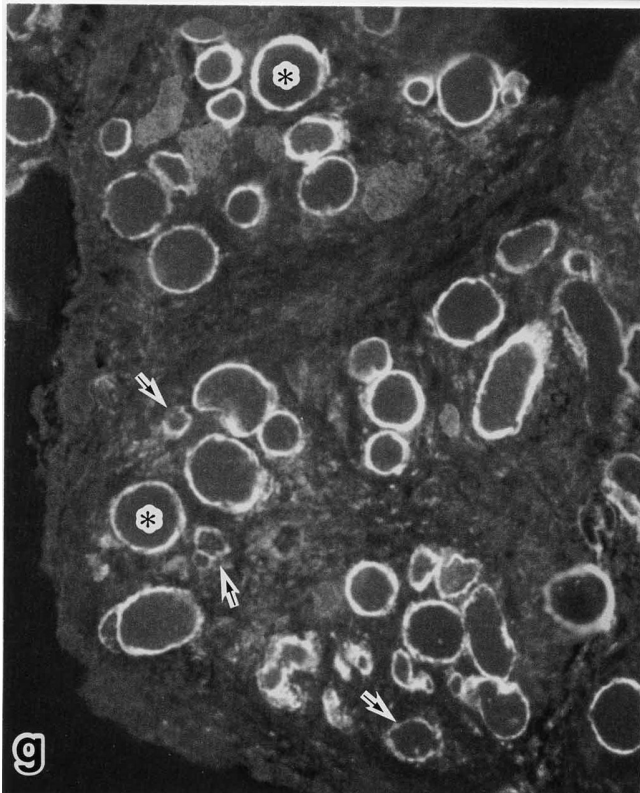
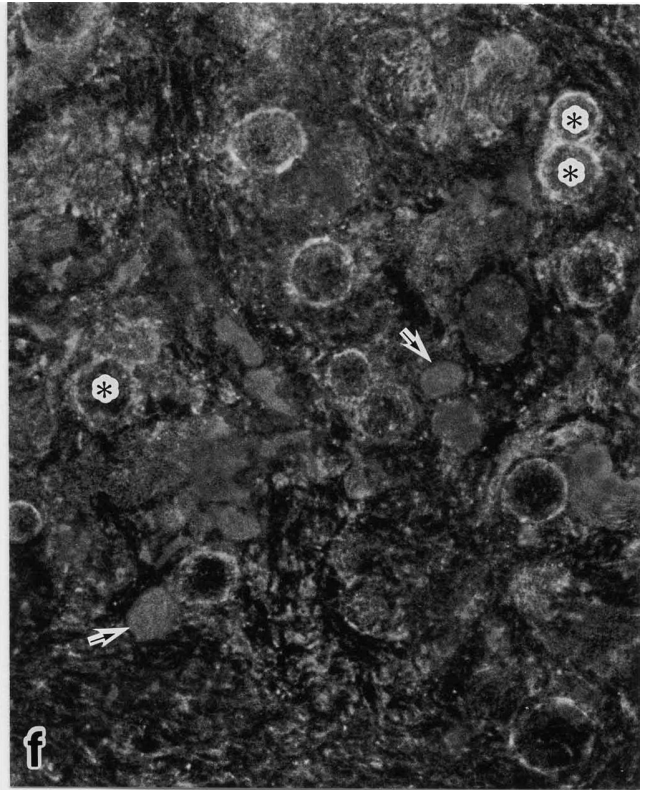
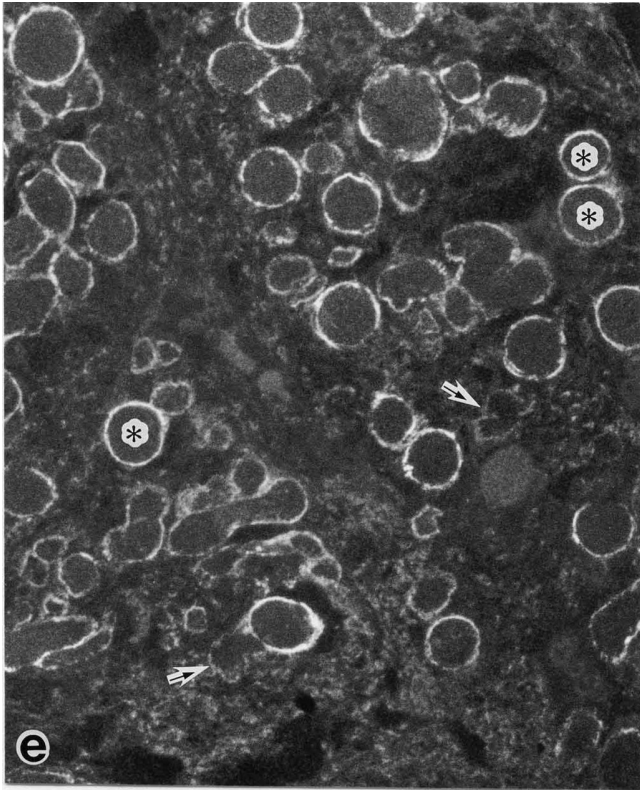


*Sarcospan in Fukuyama muscular dystrophy*

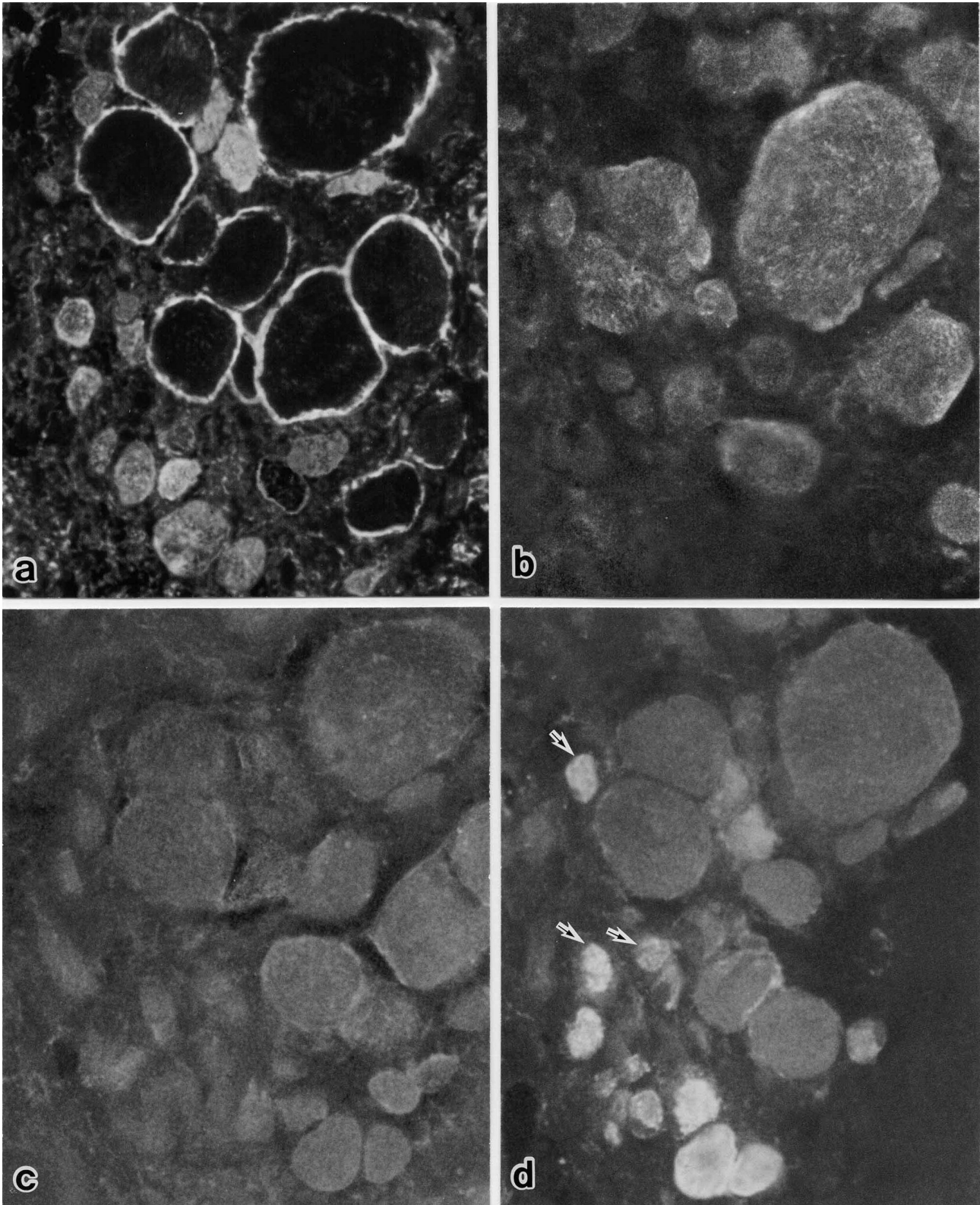


**Fig. 5.** Co-labeling immunohistochemistry with anti-sarcoglycan and sarcospan antibodies using serial FCMD muscle sections. Immunostainings with antibodies against  $\alpha$ -sarcoglycan (**a**),  $\beta$ -sarcoglycan (**c**),  $\gamma$ -sarcoglycan (**e**),  $\delta$ -sarcoglycan (**g**) and sarcospan (**b**, **d**, **f**, **h**) are shown. Sarcoglycan immunostaining preparations show the presence of continuously immunopositive, partially immunopositive and immunonegative myofibers, while sarcospan immunostaining specimens showed generally reduced expression of sarcospan at the myofiber surface. Continuously immunopositive myofibers with anti-sarcoglycan antibodies (asterisks in **a**, **c**, **e**, **g**) also expressed more or less sarcospan molecule (asterisks in **b**, **d**, **f**, **h**). Myofibers partially expressing sarcoglycan (arrows in **a**, **c**, **e**, **g**) tended to show negative immunostaining with anti-sarcospan antibody (arrows in **b**, **d**, **f**, **h**). **a-d**, x 250; **e-h**, x 290

*Sarcospan in Fukuyama muscular dystrophy*



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**Fig. 6.** Immunohistochemical stainings of serial FCMD muscle sections with anti- $\alpha$ -dystroglycan core protein (**a**), sarcospan (**b**),  $\alpha$ -dystroglycan glycosylated sugar (IIH6C4) (**c**) and neonatal myosin heavy chain (**d**) antibodies. Immunostaining with antibody against  $\alpha$ -dystroglycan core protein (**a**) shows the presence of immunopositive FCMD myofibers that tend to be large diameter myofibers (**a**), while immunostaining specimen with anti-sarcospan antibody reveals reduced expression of sarcospan (**b**). Immunohistochemical preparation with anti-glycosylated  $\alpha$ -dystroglycan antibody (IIH6C4) (**c**) shows almost total deficiency of glycosylated  $\alpha$ -dystroglycan expression. Anti-neonatal myosin antibody immunoreactivity (**d**) reveals that immunopositive myofibers (arrows in **d**) tend to be small diameter FCMD myofibers. x 550

**Table 3.** Immunonegative fiber ratio (group 3).

	anti- $\beta$ -spectrin antibody immunonegative fiber ratio	anti-sarcospan antibody immunonegative fiber ratio
	P<0.0001	
5 FCMD	15.1 $\pm$ 5.1%*	72.7 $\pm$ 4.3%*
	P<0.0001	
5 DMD	11.0 $\pm$ 2.2%#	77.6 $\pm$ 3.6%#
5 disease control (3 myotonic dystrophy + 2 facioscapulohumeral dystrophy)	0%	0%
5 normal control	0%	0%

\*: P<0.0001 by two-tailed t test; #: P<0.0001 by two-tailed t test

(p<0.0001 by the two-tailed t test) (Table 3).

#### Real-time RT-PCR

The standard curves for the quantification of human sarcospan and GAPDH mRNAs were linear across four log ranges of RNA concentration. The correlation coefficients were 0.96 for human sarcospan mRNA and 0.981 for GAPDH mRNA. Electrophoretic analysis of the real-time RT-PCR product on 2% agarose gel showed the expected 242bp band for human sarcospan cDNA and 452 bp band for GAPDH cDNA. The group mean ratios  $\pm$  standard error of the mean of human sarcospan mRNA copy number versus GAPDH mRNA copy number in FCMD, DMD and normal control muscles were 1.86 $\pm$ 1.02, 5.19 $\pm$ 2.83 and 1.62 $\pm$ 0.35, respectively. The differences of ratios between FCMD and normal control, DMD and normal control, and FCMD and DMD groups were not statistically significant (P>0.1 two-tailed t test).

#### Discussion

No cases of muscular dystrophy associated with primary mutations in the sarcospan gene have been reported (Crosbie et al., 2000), but many studies show a reduction or deficiency of sarcospan in limb girdle muscular dystrophy patients and in animal models, such as sarcoglycan knockout mice (Duclos et al., 1998; Coral-Vazquez et al., 1999; Barresi et al., 2000; Durbeej et al., 2000, 2003). The functional significance of sarcospan is unknown at present. Crosbie et al. (1997) postulated two functional possibilities of sarcospan. One of these functions is channel function, because multiple transmembrane regions of sarcospan might form a pore in the sarcolemma. Although no significant homology of tetraspanins with any other known gene families has been found, it has been suggested that some of their structural features are similar to ligand-gated ion channels (Wright and Tomlinson, 1994; Maecker et al., 1997). The second possible function of sarcospan is a solid anchorage for the rest of DGC to the sarcolemma

(Peter et al., 2007). Over 60% of sarcospan's amino acids are predicted to be within the membrane, and sarcospan's transmembrane domains are expected to hold this protein firmly within the lipid bilayer (Crosbie et al., 1997). Sarcospan is enriched at the myotendinous junction (Crosbie et al., 1999). In this context, the possible function of sarcospan is thought to be a force transmission from myofibrils to extracellular matrix through the sarcolemma. Indeed  $\beta$ -sarcoglycan is an important protein in the formation of the sarcoglycan complex associated with sarcospan, and the role of the sarcoglycan complex and sarcospan may be to strengthen the dystrophin axis connecting the extracellular basement membrane with the intracytoplasmic cytoskeleton (Araishi et al., 1999). Crosbie et al. (2000) reported that sarcospan was absent in a  $\gamma$ -sarcoglycan patient with normal levels of  $\alpha$ -,  $\beta$ - and  $\delta$ -sarcoglycan, and that the C-terminus (extracellular domain) of  $\gamma$ -sarcoglycan is critical for the functioning of the entire sarcoglycan-sarcospan complex. The  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -sarcoglycans and sarcospan molecules are ultrastructurally associated with each other and are present in a cluster (Wakayama et al., 1999, 2001; Hayashi et al., 2006). Interestingly, however, Lebakken et al. (2000) reported that sarcospan-deficient mice maintained normal muscle function and expressed other components of DGC at the sarcolemma; gross histological abnormalities of skeletal muscles from these mice were not observed and sarcospan-deficient muscles maintained normal force and power generation capabilities (Lebakken et al., 2000). The results of Lebakken et al. (2000) suggest that either sarcospan is not necessary for normal muscle function or the sarcospan-deficient muscle is compensating for the absence of sarcospan in some way, possibly by using another protein to carry out its function. However, Peter et al. (2007) reported that disrupted mechanical stability of DGC caused severe muscular dystrophy in transgenic mice overexpressing sarcospan. Therefore, the sarcoglycan-sarcospan complex may have an important role in a metabolic or signaling pathway, or in a still unidentified way (Barresi et al., 2000). Yoshida et al. (2000) showed biochemical evidence for association of dystrobrevin with the sarcoglycan-sarcospan complex and postulated the possible role of the sarcoglycan-sarcospan complex as the signaling function from its association with the intracellular signaling molecule dystrobrevin. Indeed, a substantial amount of residual  $\alpha$ 1-syntrophin and dystrobrevin is still detected in FCMD and DMD muscles (Wakayama et al., 2006).

This study clearly demonstrated that the expression of sarcospan at the protein level markedly decreased both in FCMD and DMD myofibers. We here confirmed the results of Crosbie et al. (1997), who described that sarcospan markedly decreases in DMD muscles. Although the expression of  $\beta$ -spectrin protein slightly decreased in FCMD and DMD myofibers in this study, the expression of the sarcospan molecule more severely

## Sarcospan in Fukuyama muscular dystrophy

and statistically significantly decreased in myofibers with both diseases. Nevertheless, the levels of sarcospan mRNA in FCMD and DMD muscles showed that they were not statistically different from the level of normal control muscles, although the sarcospan mRNA level in DMD muscles appeared to be higher than levels of sarcospan mRNA in FCMD and normal control muscles. Taking these results into consideration, the mechanism of the reduced expression of sarcospan in DMD and FCMD could be as follows. In DMD boys without dystrophin, the other members of DGC, including sarcospan, decrease, perhaps as the result of premature protein degradation, improper assembly of the complex, or aberrant transportation to the sarcolemma (Crosbie et al., 1997). On the other hand, a 60-kDa protein fukutin is localized in the cis compartment of the Golgi apparatus (Matsumoto et al., 2004) that is the central stage of glycosylation and is where the fukutin protein is well positioned to affect the glycosylation of  $\alpha$ -dystroglycan (Martin, 2006). Insertion of a retrotransposon into the 3'-untranslated sequence disrupts the fukutin gene, leading to decreased glycosylation of  $\alpha$ -dystroglycan in FCMD muscles (Kobayashi et al., 1998; Toda et al., 2003; Barresi and Campbell, 2006; Percival and Froehner, 2007). Severely reduced expression of  $\alpha$ -dystroglycan is due to impaired glycosylation in FCMD muscles (Toda et al., 2003). The deficiency of glycosylated  $\alpha$ -dystroglycan expression compared with the relative expression of  $\alpha$ -dystroglycan core protein in FCMD muscles was also observed in this study.  $\alpha$ -Dystroglycan is approximately half carbohydrate by molecular weight and this characteristic is attributed to the presence of a serine-threonine (S-T)-rich mucin domain in the middle of the protein that contains up to 55 sites of O-linked glycosylation. The O-linked carbohydrates are attached to proteins by means of an S or T residue. The decreased staining pattern of sarcoglycans and sarcospan of FCMD muscles in this study is attributed to the likely loss of O-linked glycosylation in the Golgi apparatus of FCMD muscles. Alternatively glycosylated  $\alpha$ -dystroglycan deficiency may affect the molecular architecture of basal lamina of FCMD myofibers, although merosin expression was nearly normal, and may in turn lead to decreased expression of sarcoglycan, and finally sarcospan, at the FCMD myofiber surfaces. If the forced expression of the glycosylated  $\alpha$ -dystroglycan of FCMD muscles leads to increased expression of sarcoglycans and sarcospan, our proposed hypothetical mechanism of the reduced expression of these molecules in FCMD muscles will be justified. In this study, we showed severely reduced expression of sarcospan in muscles with FCMD and DMD. Although the functional significance of sarcospan is still speculative, if forced expression therapy of sarcospan in the muscular dystrophies with severe muscle weakness becomes available in the future, and this treatment provides the affected children with the muscle strength, the treatment will throw light into the functional significance of

sarcospan.

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*Sarcospan in Fukuyama muscular dystrophy*

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