Background: In Duchenne muscular dystrophy (DMD), previous freeze-fracture electron microscopic studies demonstrated that muscle plasma membrane contained markedly decreased numbers of orthogonal arrays. Recent investigations showed that orthogonal arrays were composed of aquaporin 4 (AQP4) molecules, a member of the water channel protein family.

Objectives: To study whether the immunostainability of anti-AQP4 antibody is reduced in muscles of patients with DMD and whether, if it is reduced, the problem is at the genomic DNA, messenger RNA (mRNA), or post-transcriptional level.

Patients and Methods: We analyzed the muscle and blood samples from 6 boys with DMD, 6 normal control subjects, and 12 patients with neuromuscular diseases at the protein, genomic DNA, and mRNA levels. At the protein level, immunohistochemical staining and immunoblot analysis were performed. At the genomic DNA and mRNA levels, the polymerase chain reaction and reverse transcription polymerase chain reaction, respectively, were used to screen for mutations in the AQP4 gene.

Results: At the protein level, immunohistochemical staining of our originally generated rabbit anti-AQP4 antibody in DMD muscles was markedly reduced. Most of the DMD myofibers showed negative staining with sporadic partially positive fibers at their myofiber surface, whereas the control muscles displayed continuous myofiber surface staining. Immunoblot analysis showed that the content of AQP4 in DMD muscles was remarkably decreased. Amplification of leukocyte genomic DNA by polymerase chain reaction showed that the patients with DMD had genomic DNA of the AQP4 molecule. Quantitative reverse transcription polymerase chain reaction demonstrated that DMD skeletal muscles contained markedly decreased AQP4 mRNA compared with controls.

Conclusion: The reduction in AQP4 in DMD muscles results from decreased levels of AQP4 mRNA in DMD myofibers.

Arch Neurol. 2002;59:431-437
MATERIALS AND METHODS

MUSCLE AND BLOOD SAMPLES

Biopsy specimens of quadriceps femoris muscles were obtained with the patient under local anesthesia from 6 boys with DMD whose ages at muscle biopsy ranged from 1 year 6 months to 8 years 5 months. They had proximal muscle weakness, atrophy in various degrees and calf pseudo-hypertrophy. All patients with DMD had markedly high serum creatine kinase levels, ranging from 10960 to 20610 U/L at the time of muscle biopsy. The examination of leukocyte genomic DNA from 2 of 6 patients with DMD demonstrated the dystrophin gene deletion at exons 8, 12, 13, 17, and 19 in one boy and exons 49 through 52 in another boy. All muscles from patients with DMD showed negative dystrophin immunostaining. For normal control specimens, 6 histochemically normal biopsy specimens of quadriceps femoris muscles were obtained from patients who were deemed to have myopathy but were free of neuromuscular disorders after histochemical and immunologic examinations. Muscle specimens obtained by biopsy from 5 patients with myotonic dystrophy, 4 patients with neurogenic muscle atrophy, 2 patients with limb girdle muscular dystrophy, and 1 patient with myasthenia gravis served as disease controls.

The leukocyte genomic DNA–coding AQP4 molecule was analyzed in blood samples of 6 patients with DMD, 6 healthy control subjects, and 5 patients with myotonic dystrophy, 4 patients with neurogenic atrophy, 2 patients with limb girdle muscular dystrophy, and 1 patient with myasthenia gravis. In this study, blood and muscle samples were taken from patients under informed consent.

RESULTS

IMMUNOBLOT ANALYSIS OF ANTIBODY

Immunoblot analysis showed that the antibodies against AQP4, dystrophin, and β-spectrin reacted with 31-, 427-, and 270-kd protein extracts, respectively, of normal and disease control human quadriceps femoris muscles (Figure 1). The reactions for AQP4 and β-spectrin were markedly and slightly decreased, respectively, in DMD muscle extracts (Figure 1A, C), whereas the reaction for dystrophin was absent in DMD muscle extract (Figure 1B).

Peptide synthesis of AQP4 and antibody production

General procedures for peptide synthesis and antibody generation were similar to those described previously. Briefly, the peptide (C-EKKGKDSSGELSSV) of the C-terminal end of the cytoplasmic domain in the rat AQP4 molecule was synthesized and extra cysteine was added to the N-terminus of this peptide. Bovine thyroglobulin was added at an extra cysteine residue. The antibody against this peptide was generated in rabbits. Solid-phase enzyme-linked immunosorbent assay was used to determine the rabbit polyclonal antibody titer, which was X204800.

IMMUNOHISTOCHEMISTRY

The muscle biopsy specimens were immediately frozen in isopentane cooled with liquid nitrogen. Frozen, 6-µm-

IMMUNOBLOT ANALYSIS OF ANTIBODY

Immunoblot analysis of antisera against AQP4 in the histochemically normal specimens of human quadriceps femoris muscles and those of patients with DMD was performed with a previously described method with minor modifications. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with a 12.3% homogeneous gel for AQP4, and 3% to 10% gradient gel for dystrophin and β-spectrin. The protein was transferred from gel to a clear blot P membrane sheet (ATTO, Tokyo, Japan) by horizontal electrophoresis at 108 mA for 90 minutes at room temperature. Immunostainings were done with rabbit anti-AQP4 and anti-β-spectrin antisera diluted 1:500 and 1:200, respectively, and with mouse monoclonal anti–dystrophin (Dys 1) antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, England) diluted 1:500.

POLYMERASE CHAIN REACTION AMPLIFICATION OF GENOMIC DNA

The PCR amplification of leukocyte genomic DNA showed that all patients with DMD and all healthy control subjects and disease control patients had genomic DNA of human AQP4 as a single band (Figure 3).
thick cross sections of the muscles were placed on coverslips and incubated with primary antiseraum, diluted 1:200 for rabbit anti-AQP4. Serial cross sections of muscles were also incubated with primary monoclonal anti-Dys 1 antibody (Novocastra) diluted 1:30 and rabbit anti-β-spectrin antiseraum13 diluted 1:200. Indirect immunofluorescent staining was performed according to the method previously described.14

POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) was performed on genomic DNA extracted from DMD, control, and disease control leukocytes with a kit (TaKaRa Ex Taq [code RR017]; TaKaRa Co, Kyoto, Japan) according to the manufacturer’s protocol. The oligonucleotide primers were designed from human AQP4 sequence: sense strand, 5'-CTCAGATTGCAACCATG-3' , and antisense strand, 5'-GGATTCCTGCTCCAATGA-3'. The PCR amplification was performed by denaturing the genomic DNA at 94°C for 5 minutes and conducted by 30 cycles of 30 seconds at 94°C and 52°C and 1 minute at 72°C. The reaction mixture contained 1× PCR buffer, 2mM magnesium chloride, 0.8mM of deoxyribonucleoside 5'-triphosphate (dNTP), 0.8µM each of primer pair, and 0.1-U/µL Ex Taq polymerase. The PCR products were observed by means of 2% agarose gel electrophoresis.

QUANTITATIVE REVERSE TRANSCRIPTION PCR

Total RNA was extracted from approximately 30 mg of each DMD, control, or disease control muscle sample with an acid phenol extraction reagent (TRiZol [code 15596-026]; Gibco BRL, Rockville, Md). The concentration of AQP4 mRNA was estimated by quantitative reverse transcription PCR (RT-PCR). The oligonucleotide primers were designed from human AQP4 sequence: sense strand (AQP4F), 5'-GGTGCTCATCTCCCCCTTGCTTTT-3'; antisense strand (AQP4R), 5'-GTCTTTCCCCCTCCTCCTCTC-3'. The synthetic DNA competitor was made by PCR from synthetic DNA templates provided in the kit (Competitive DNA Construction Kit [code RR017]; TaKaRa Co), which included both the upper (AQP4F) and lower (AQP4R) sequences specific for human AQP4. We used a kit (Competitive RNA Transcription Kit [code 6125]; TaKaRa Co) to make the synthetic RNA competitor from the synthetic DNA template by transcription with SP6 RNA polymerase. The concentration of purified AQP4 RNA competitor was calculated as a copy number per microgram of total RNA. Serial dilutions of synthetic AQP4 RNA competitor were prepared. With 50 ng of total RNA of each sample and serial dilutions of synthetic AQP4 RNA competitor, several rounds of competitive RT-PCR for each sample were carried out according to the kit instruction (mRNA Selective PCR Kit [code RR025A]; TaKaRa Co). Both the sample and the competitor RNA were reverse transcribed for 30 minutes at 50°C followed by 25 cycles of PCR (30 seconds each at 85°C and 60°C and 1 minute at 72°C) with the reaction mixture containing 1× PCR buffer, 0.4pM each of primer pair, 5mM magnesium chloride, 1mM each of dNTP analogue mixture, 0.8-U/µL ribonuclease inhibitor, 0.1-U/µL reverse transcriptase, and 0.1-U/µL Taq polymerase. The competitive RT-PCR products were separated by electrophoresis, and the concentration of AQP4 mRNA in the sample was estimated by image analysis with an image processing system (NIH image 1.61; National Institutes of Health, Bethesda, Md). To compare the concentration of AQP4 mRNA with that of a housekeeping gene, a parallel assay of β-actin was performed for each sample by using human β-actin Competitive PCR Set (code 6607) TaKaRa Co).

Figure 1. Immunoblot analysis of aquaporin 4 (AQP4) (A), dystrophin (B), and β-spectrin (C) expression in extracts of histochemically normal human quadriceps femoris muscles (lane 1) and muscles of patients with Duchenne muscular dystrophy (DMD) (lane 2). Electrophoresis and blotting were performed as described in the “Materials and Methods” section. In normal human muscle extracts, the bands of AQP4, dystrophin, and β-spectrin were observed at 31, 427, and 270 kd, respectively (A, B, and C, respectively). The reactions for AQP4 and β-spectrin were markedly and mildly decreased in DMD muscle extracts, respectively (A and C, respectively), whereas the reaction for dystrophin was absent in DMD muscle extract (B). Numbers to the left in each figure indicate molecular masses of standards.
Figure 2. A through C, Immunohistochemical staining of normal muscle with anti-aquaporin 4 (anti-AQP4) (A), anti-dystrophin (B), and anti-β-spectrin (C) antibodies. The cell surface of each myofiber showed a thin layer of immunofluorescence with these antibodies. No immunostaining of intracellular structures was noted. D through F, In muscle samples from patients with Duchenne muscular dystrophy (DMD), immunostaining for AQP4 was markedly reduced and the pattern of staining of AQP4 in the DMD muscles was strikingly patchy, with some large fibers having normal amounts and almost all small fibers being negative (D). The immunoreaction with anti-dystrophin antibody at the DMD myofiber surface was negative (E), whereas that with anti-β-spectrin antibody showed positive staining (F). The myofibers with partial positive staining for anti-AQP4 antibody at their surface membranes (D) showed negative staining with anti-dystrophin antibody (E) (original magnification ×330).
QUANTITATIVE RT-PCR STUDY

The gel electrophoresis of competitive RT-PCR product of AQP4 in patients with DMD (Figure 4A), healthy control subjects (Figure 4B), and disease control patients contained 2 bands of 733 and 568 base pairs (bp), which corresponded to the RT-PCR products of total RNA samples extracted from muscle biopsy specimens and AQP4 RNA competitor, respectively. The markedly decreased 733-bp RT-PCR products of AQP4 were observed with 50 ng of total RNA from 6 patients with DMD as compared with those of healthy subjects and disease control patients. The gel electrophoresis of competitive RT-PCR products of β-actin in patients with DMD (Figure 4C), healthy subjects (Figure 4D), and disease control patients included 2 bands of 275 and 365 bp, which corresponded to the RT-PCR products of total RNA samples extracted from muscle biopsy specimens and β-actin RNA competitor, respectively. The 275-bp RT-PCR products of β-actin appeared to be normal in 3 of 6 patients with DMD as compared with those of healthy subjects and disease control patients.

The group mean concentration of AQP4 mRNA in DMD muscles by quantitative RT-PCR was 82 million molecules per microgram of total RNA, whereas that in normal and disease control muscles was 3962 and 3404 million molecules per microgram of total RNA, respectively. The group mean concentration of β-actin mRNA in DMD muscles by quantitative RT-PCR was 5458 million molecules per microgram of total RNA, whereas that in normal and disease control muscles was 16236 and 18066 million molecules per microgram of total RNA, respectively (Figure 5). To compare AQP4 mRNA of...
DMD muscles with that of normal control muscles, the ratio of copy number of AQP4 mRNA to copy number of β-actin mRNA was calculated in patients with DMD, healthy subjects, and disease control patients. The ratios were (26.1±8.4) \times 10^{-3} \text{(group mean±SEM)}, (247.5±65.4) \times 10^{-3}, and (261.7±71.2) \times 10^{-3}, respectively. The ratio in DMD muscles was statistically significantly lower than that in normal \( (P<.01) \) and disease control \( (P<.05) \) muscles.

**COMMENT**

Duchenne muscular dystrophy is an X-linked recessive muscle-wasting disorder caused by the mutation of the dystrophin gene,\(^{13}\) which produces the membrane-associated cytoskeletal protein with 427-kd molecular weight.\(^{10}\) Before the discovery of dystrophin and its causative role in DMD, Scotland et al.\(^{17}\) and Wakayama et al.\(^{18}\) conducted freeze-fracture electron microscopic studies of the muscle plasma membrane in DMD in accordance with the membrane theory of DMD\(^{19}\) and membrane defects in DMD.\(^{20}\) They found marked depletion of OA particles in the muscle plasma membrane P face and of OA pits in the muscle plasma membrane E face of DMD,\(^{1,2,18}\) in addition to reduced individual intramembranous particles in this disease. Recently, OA particles have been proved to consist of AQP4 molecule.\(^{7,9}\) At present, the relationship between OA and dystrophin molecule is unknown. However, the indirect association of AQP4 molecule with dystrophin was suggested via the modular protein-protein interaction domain (PDZ domain)\(^{21}\) of α-1-syntrophin,\(^{22,23}\) which is the 59-kd intracellular dystrophin-associated protein\(^{22}\) and whose expression is reduced in DMD\(^{24}\) and mdx mice.\(^{25}\) In fact, the immunoreactivity of rabbit anti-AQP4 antibody was markedly reduced in the sarcolemma and the vascular foot membrane of brain astrocytes in α-1-syntrophin null mice,\(^{23}\) and AQP4 mRNA was detected in soluble fractions but not in the membrane-associated fraction of α-1-syntrophin null mice.\(^{23}\)

In our group's freeze-fracture studies of DMD muscle plasma membrane,\(^{2,18}\) we noticed that most of the DMD myofiber plasma membranes contained a markedly reduced density of OA particles; however, some of the DMD myofibers contained the normal density of OA particles at their plasma membranes. The results of the present immunohistochemical study with anti-AQP4 antibody in DMD muscles coincided well with those of freeze-fracture studies of DMD muscles, since the sporadic partially positive–staining large fibers with anti-AQP4 antibody were noted in DMD muscles. We were interested in whether the immunoreactivity of these AQP4-positive DMD myofibers was also positive for the anti-dystrophin antibody. However, the serial cross sections of DMD myofibers showed that the AQP4-positive fibers displayed the negative dystrophin immunostaining.

In the next step, we investigated the mechanism of the depletion of AQP4 molecule in DMD muscles at the genomic DNA and mRNA levels. As expected, the PCR study disclosed that the genomic DNA of human AQP4 molecule was present normally in DMD leukocytes and the decrease of AQP4 molecule in DMD muscles was not due to the mutation of genomic DNA. In addition, the mRNA of human AQP4 molecule was present in DMD muscles. The AQP4 staining pattern of DMD muscles with some large fibers being positive and almost all small fibers being negative suggested a secondary rather than a primary change. However, quantitative RT-PCR showed that the amount of mRNA of AQP4 was markedly decreased in DMD muscles. With regard to other membrane cytoskeletal proteins in DMD muscles, the contents of dystroglycans and sarcoglycans have been reported to be decreased.\(^{24,26-28}\) The contents of mRNA for these proteins of DMD muscles were examined in β-dystroglycan and α-sarcoglycan; they were described to be normal in dystroglycan\(^{26}\) and slightly decreased in α-sarcoglycan,\(^{27}\) although the mRNA levels of other components of sarcoglycans such as β-, γ-, and δ-sarcoglycans have not been reported so far.\(^{27}\) This study clarified that the depletion of AQP4 molecule at the DMD muscle plasma membrane was due to the decrease of mRNA of human AQP4 in DMD muscles. The reduced content of AQP4 mRNA in DMD muscles implies either decreased transcription or increased degradation of the message. The altered motor nerve influence on the DMD muscles might have some relationship to decreased transcription. In fact, the dystrophin protein of 116 kd (Dp116) is present in normal peripheral nerve,\(^{29}\) but this dystrophin short form is absent in DMD, resulting in the altered function of motor innervation in DMD muscles. The abnormal innervation of DMD myofibers might be also brought about by the proliferated connective tissue of DMD muscles. In addition, the freeze-fracture study of the experimentally denervated regenerating myofibers showed no OA particles at their muscle plasma membrane P face,\(^{30}\) and the mRNA of AQP4 molecule was absent in these muscles.\(^{31}\) Further investigations on the mechanism of the decrease of AQP4 mRNA in DMD muscles will throw light onto the mechanism of the depletion of OA particles, DMD muscle membrane lysis causing high serum levels of sarcoplasmic enzymes and the formation of delta lesions of DMD myofibers, and finally the dysfunction and degeneration of DMD muscles.

**Figure 5.** Quantitative reverse transcription polymerase chain reaction study of aquaporin 4 (AQP4) messenger RNA (mRNA). In muscles from patients with Duchenne muscular dystrophy (DMD), AQP4 mRNA level was markedly decreased. In contrast, AQP4 mRNA was expressed in normal control and disease control muscles at the same level. The level of β-actin mRNA in muscles of patients with DMD was about one third that of normal control and disease control muscles.
Accepted for publication September 12, 2001.

Author contributions: Study concept and design (Dr Wakayama); acquisition of data (Drs Wakayama, Jimi, Inoue, Murahashi, Kumagai, Yamashita, Hara, and Shibuya and Ms Kojima); analysis and interpretation of data (Drs Wakayama and Jimi); drafting of the manuscript (Drs Wakayama, Jimi, Inoue, Murahashi, Kumagai, Yamashita, Hara, and Shibuya and Ms Kojima); critical revision of the manuscript for important intellectual content (Drs Wakayama and Jimi); statistical expertise (Drs Wakayama and Jimi); obtaining funding (Dr Wakayama); administrative, technical, or material support (Drs Wakayama, Jimi, Inoue, Murahashi, Kumagai, Yamashita, Hara, and Shibuya and Ms Kojima); study supervision (Dr Wakayama).

We thank Yoko Matsuzaki, MP, for her skillful technical assistance.

Corresponding author and reprints: Yoshihiro Wakayama, MD, PhD, Department of Neurology, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama 227-8501, Japan (e-mail: wakayama@showa-university-fujigaoka.gr.jp).

REFERENCES


