SOMATIC CELL HETEROGENEITY BETWEEN DNA EXTRACTED FROM LYMPHOCYTES AND SKELETAL MUSCLE IN CONGENITAL MYOTONIC DYSTROPHY

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Summary Myotonic dystrophy (DM) results from the expansion of an unstable CTG trinucleotide repeat in the 3' untranslated region of mRNA encoding a putative serine/threonine protein kinase. The degree of the CTG repeat amplification in genomic DNAs extracted from lymphocytes correlates with disease severity. We have analyzed the amplification of the CTG repeat of DNAs extracted from skeletal muscles and lymphocytes in five congenital DM patients. The amplification from skeletal muscles showed an increase of about 1.5 kb to 3.5 kb larger than that from lymphocytes in all patients. Furthermore, we have investigated the somatic instability of the CTG repeat in various tissues from a severe congenital DM patient.

Key Words myotonic dystrophy (DM), congenital form, unstable trinucleotide (CTG) repeat somatic cell heterogeneity

INTRODUCTION

Myotonic dystrophy (DM) is an autosomal dominant, multisystemic disorder characterized by myotonia, progressive muscle weakness and atrophy, cardiac conduction disturbances and cataracts (Harper, 1989). DM shows marked variability in its expression, ranging from severe congenital forms to an asymptomatic form. The rare congenital form of DM is associated with severe hypotonia, feeding difficulty, neonatal respiratory distress, motor developmental delay, and mental retardation. Höweler *et al.* (1989) reported that transmission of congenital DM is exclusively maternal. Patient's mothers frequently have mild to subclinical manifestations of DM. Ashizawa *et al.* (1993a) observed anticipation, successively

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earlier age at onset and increasing severity of the disease in successive generations in the pedigree of a DM family. However, we have found genetic evidence confirming the original hypothesis of paternal origin of congenital DM, and have suggested an explanation for the phenomenon of anticipation (Ohya *et al.*, 1994).

The molecular basis of DM mutation is an unstable trinucleotide (CTG) repeat, located in the 3' end of a transcript encoding a putative serine/threonine protein kinase gene of chromosome 19q13.3 (Brook *et al.*, 1992; Mahadevan *et al.*, 1992; Fu *et al.*, 1992). Trinucleotide repeat mutation similar to DM have been identified in Fragile X mental retardation syndrome (Verkerk *et al.*, 1991), spinal and bulbar muscular atrophy (Kennedy's disease) (La Spada *et al.*, 1991), FRAXE mental retardation (Knight *et al.*, 1993), Huntington's disease (The Huntington's disease collaborative research group, 1993), spinocerebellar ataxia type 1 (Orr *et al.*, 1993), dentatorubral-pallidoluysian atrophy (Nagafuchi *et al.*, 1994), and Machado-Joseph disease (Kawaguchi *et al.*, 1994).

The disease severity in DM generally correlates to the size of the expanded CTG repeats in lymphocytes from patients with DM, but the mechanism of this is unclear. In adult DM patients, the expansion of the repeat isolated from skeletal muscle is larger than that in lymphocytes from the same patients. In this paper we described a comparison of the CTG repeat amplification of genomic DNAs from lymphocytes and skeletal muscles in five unrelated cases of congenital DM patients, based on Southern blot analysis using the probe p5B1.4 (Shelbourne *et al.*, 1992) derived from cDNA25 (Buxton *et al.*, 1992). Furthermore, we have examined the genomic DNAs of various tissues in a severely affected neonate with congenital DM and demonstrated somatic cell heterogeneity in the organ tissues of congenital DM.

MATERIALS AND METHODS

Materials. We studied five patients with congenital DM whose mother had been diagnosed with adult DM. These congenital patients had characteristic clinical features such as marked hypotonia with facial weakness, respiratory distress, and feeding difficulty during the neonatal period. In addition to the typical clinical manifestations of congenital DM, muscle histopathology was consistent with congenital DM. Table 1 summarizes the characteristics of the patients.

Peripheral blood lymphocytes from the patients were taken at the time of this study, but the muscle samples had been taken 1 to 8 years earlier, frozen in liquid nitrogen and stored at -80°C until the analyses reported here. All muscle samples were taken from the quadriceps femoral muscle. We obtained seven kinds of postmortem tissues, including liver, diaphragm, urinary bladder, small intestine, tongue, uterus, and cerebral cortex, from a severely affected neonate with congenital DM, who died of respiratory failure at one month of age.

Southern blot analysis. Genomic DNAs were prepared from peripheral-blood

Table 1. Summary of clinical features in five patients with congenital DM.

Case	1	2	3	4	5
Age (years)	2	17	3	3	5
Age diagnosed with congenital DM	1 month	9 years	2 years	2 years	1 month
Sex	F	F	F	F	M
Gestational age (weeks)	35	40	38	39	38
Weight at birth (g)	2,238	3,450	3,198	2,538	2,720
Polyhydramnios	+	—	—	—	—
Asphyxia	+	+	+	+	+
Neonatal respiratory distress	+	+	+	+	+
Period for mechanical ventilation	30 days	—	—	—	10 days
Feeding difficulty	+	+	+	+	+
Floppiness in neonatal period	+	+	+	+	+
Facial weakness	+	+	+	+	+
Mental retardation	+	+	+	+	+
Myotonia	—	+	—	—	+
Muscle weakness	+	+	+	+	+
Origin of DM gene	mother	mother	mother	mother	mother
Biopsied and diagnosed age	1 month	9 years	2 years	2 years	1 month

lymphocytes and biopsied skeletal muscle from the five patients by standard procedures (Sambrook *et al.*, 1989). Genomic DNAs were digested with the restriction endonucleases *EcoRI* and *BglI*, separated by electrophoresis on 0.8% agarose gel, and transferred onto nylon membranes using a vacuum transfer apparatus. After prehybridization, digested DNAs were hybridized to radiolabeled p5B1.4 for 24 hr. The filters were washed to a stringency of $0.1 \times \text{SSC}$ containing 0.1% SDS and autoradiography was performed for 2 to 5 days at -80°C . The probe p5B1.4, which was supplied by Dr. Keith Johnson, is a 1.4 kb *BamHI* fragment from cDNA-25 subcloned into pBluescript SK+; it was labeled by random priming.

PCR amplification method. In determining the precise number of CTG repeat of normal allele, polymerase chain reaction (PCR) amplification was carried out using CTG region flanking primers 406 and 409 reported by Mahadevan *et al.* (1992). PCR cycling conditions were as follows: initial denaturation at 95°C for 3 min, then 35 cycles of 95°C for 1.5 min, 65°C for 1 min, 72°C for 2 min, and 72°C for 7 min.

RESULTS

Southern blot analysis using *EcoRI*-digested DNAs probed with p5B1.4 showed an *EcoRI* polymorphism with alleles of 9.8 kb and 8.6 kb in normal populations

(Fig. 1). Patient 1 had alleles of 8.6 kb/15.0 kb (about 1,700 CTG repeats) in lymphocytes and 8.6 kb/16.5 kb (about 2,200 CTG repeats) in skeletal muscle. Patient 3 had alleles of 8.6 kb/15.5 kb (about 1,900 CTG repeats) in lymphocytes and 8.6 kb/17.5 kb (about 2,500 CTG repeats) in skeletal muscle. Patient 4 had alleles of 8.6 kb/14.0 kb (about 1,400 CTG repeats) in lymphocytes and 8.6 kb/17.5 kb (about 2,500 CTG repeats) in skeletal muscle. Patient 5 had alleles of 9.8 kb/14.0 kb (about 1,400 CTG repeats) in lymphocytes and 9.8 kb/15.5 kb (about 1,900 CTG repeats) in skeletal muscle. Patients 1, 3, 4, and 5 had larger expanded bands, ranging from 1.5 kb to 3.5 kb, in skeletal muscle than in lymphocytes.

Southern blot analysis using *Bgl*/I-digested DNAs probed with p5B1.4 shows a single band of 3.4 kb in normal populations. In *Bgl*/I-digested fragments (Fig. 2), patients 1, 2, 3, and 4 had additional expanding bands ranging from 4.0 kb to 5.5 kb in lymphocytes and 5.5 kb to 7.5 kb in skeletal muscle. All five patients had a larger expanded band, ranging from 1.5 kb to 3.5 kb, in skeletal muscle than in lymphocytes, which suggested that the somatic cell heterogeneity between skeletal muscle and lymphocytes was established during the early stage of childhood.

A patient with severe congenital DM had an abnormal enlarged and blurred band of 12.4 kb which demonstrated slight somatic cell heterogeneity in different organs. The tissues also showed similarity in numbers of CTG repeats (Fig. 3).

The number of CTG repeats of normal allele in lymphocytes and skeletal

Fig. 1. The expansion of the CTG repeat in *Eco*RI fragments from lymphocytes (L) and skeletal muscles (M) in four patients with congenital myotonic dystrophy (CDM). Southern blot analysis using *Eco*RI fragments probed with p5B1.4 showed an *Eco*RI polymorphism with alleles of 9.8 kb and 8.6 kb in normal populations. Sites of alleles in lymphocytes and skeletal muscle were as follows: Patient 1: 8.6 kb/15.0 kb (L), 8.6 kb/16.5 kb (M), Patient 3: 8.6 kb/15.5 kb (L), 8.6 kb/17.5 kb (M); Patient 4: 8.6 kb/14.0 kb (L), 8.6 kb/17.5 kb (M); Patient 5: 9.8 kb/14.0 kb (L), 9.8 kb/15.5 kb (M). Patients 1, 3, 4, and 5 had a larger expanded band (arrowheads), ranging from 1.5 kb to 3.5 kb, in skeletal muscle than in lymphocytes.

Fig. 2. The expansion of the CTG repeat in *Bgl*II fragments from lymphocytes (L) and skeletal muscles (M) in four patients with congenital myotonic dystrophy (CDM). Southern blot analysis using *Bgl*II fragments probed with p5B1.4 showed a single band of 3.4 kb in normal populations. Sites of alleles in lymphocytes and skeletal muscle were as follows: Patient 1: 3.4 kb/8.5 kb (L), 3.4 kb/10.0 kb (M); Patient 2: 3.4 kb/9.0 kb (L), 3.4 kb/11.0 kb (M); Patient 3: 3.4 kb/7.5 kb (L), 3.4 kb/11.0 kb (M); Patient 4: 3.4 kb/7.5 kb (L), 3.4 kb/11.0 kb (M). Patients 1, 2, 3, and 4 had a larger expanded band (arrowheads), ranging from 1.5 kb to 3.5 kb, in skeletal muscle than in lymphocytes.

muscle was identical by PCR analysis (data not shown).

DISCUSSION

Myotonic dystrophy (DM) is quite variable in phenotype, ranging from a severe congenital form that is frequently fatal soon after birth to a complete absence of symptoms. The clinical manifestations of congenital DM are quite distinct from these of the adult form. The genetic mutation underlying DM is an unstable trinucleotide (CTG) repeat, recently located in the 3' end of a transcript encoding a putative serine/threonine protein kinase gene (Brook *et al.*, 1992; Mahadevan *et al.*, 1992; Fu *et al.*, 1992). Since then Fu *et al.* have also found that patients with adult DM have decreased levels of messenger RNA (mRNA) and protein expression (Fu *et al.*, 1993). Alternative spliced forms of mRNAs have been identi-

Fig. 3. The expansion of the CTG repeat in *Bgl*I fragments from various tissues in an autopsied patient with severe congenital myotonic dystrophy (CDM). 1, skeletal muscle; 2, cardiac muscle; 3, liver; L, liver; D, diaphragm; U, urinary bladder; I, small intestine; T, tongue; Ut, uterus; C, cerebral cortex; 4, lymphocyte. 1, 2, 3, 4: normal individuals. The patient with severe congenital DM had an abnormal enlarged and blurred band (an arrowhead) of 12.4 kb. See text for correctionary.

fied in various tissues of DM (Jansen *et al.*, 1992; Fu *et al.*, 1993), suggesting that the product of the gene as it is expressed in different tissues could influence the clinical features of DM. Although the molecular analyses of DM has developed rapidly, the mechanism of DM gene function is still unknown.

The disease severity of congenital DM generally seems to correspond to the length of CTG repeat amplification in mutant genes from lymphocytes, but this correlation is weaker in the alleles of other organs such as skeletal muscle. Anvret *et al.* (1993) were the first to report that the expansions of CTG repeat in muscle from patients with adult form of DM were much larger than those in lymphocytes. They demonstrated that the expansions seen in muscle from adult DM were stable over a significant time period (10–15 years) and were useful to predict the progression of DM symptoms. Thornton *et al.* (1994) reported that the DM-specific expansive alleles in skeletal muscle from adult DM were 2- to 13-fold the size of those in lymphocytes; different muscles were generally consistent with each other within each patient. Ashizawa *et al.* (1993b) also reported more expanded CTG repeat in skeletal muscle from adult DM patients than lymphocytes.

Thornton *et al.* and Ashizawa *et al.* demonstrated that the size of CTG repeat amplifications in affected tissues, such as skeletal muscle, correlated better with DM severity than the repeat in lymphocytes did. They concluded that the somatic instability of the repeat might cause substantial somatic cell heterogeneity. Mahadevan *et al.* (1992) suggested that a blurred or smeared appearance of expanded alleles seen in Southern blots in lymphocytes indicated somatic cell heterogeneity

in the size of the expanded alleles. Lavedan *et al.* (1993) reported that, as observed in lymphocytes, various tissues of a 20-wk-old fetus carrying the DM mutation showed discrepancy in the size of expanded CTG repeat under Southern blot analysis, reflecting the mitotic instability in all fetal tissues.

Our data are also consistent with the results of Thornton *et al.* and Ashizawa *et al.*, and demonstrate that the extent of the expanded CTG repeat in skeletal muscle from congenital DM patients was always larger than that of in lymphocytes. It is likely that somatic cell heterogeneity is established during the early stage of childhood. Furthermore, we displayed somatic cell heterogeneity within different organs except for lymphocytes from the same congenital DM patient, and minimal degree disparity among the expansions of CTG repeats of various tissues. The mechanism of the DM gene is still unknown. A next topic is to analyze the DM kinase gene on the mRNA and protein level.

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