

Decreased Expression of Alpha-B-crystallin in C2C12 Cells that Express Human DMPK/160CTG Repeats

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Abstract

The effect of oxidative stress on myogenic cells with expanded CTG repeats in the myotonic dystrophy protein kinase (DMPK) gene was investigated using DMPK cDNA-transformants in order to investigate the disease process underlying myotonic dystrophy. It was reported that DM model cell was highly sensitive against oxidative stress. In this study, we investigated the viability to hydrogen peroxide or menadione of DM model C2C12 cells, which were stably transfected with DMPK cDNA containing 160 CTG repeats in 3'untranslated region. We found that cells containing 160 CTG repeats were more sensitive to oxidants than the cells containing 5 CTG repeats. We confirmed that the mutant cells died with low concentrations of the oxidant, compared with that of control. Next, total RNA was isolated from these cell cultures and Northern blot analysis was performed. Decrease in the expression of alpha-B-crystallin was observed in C2C12 with longer CTG repeats. These results suggest that expanded CTG repeats in DMPK increase the susceptibility of cells to oxidative stress by suppressing chaperon genes expression.

Keywords: myotonic dystrophy, oxidative stress, C2C12 cells, DMPK, CTG repeat

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Myotonic dystrophy (DM), the most common muscular dystrophy in adults, is characterized by hyperexcitability of skeletal muscle (myotonia), muscle degeneration (myopathy), a conduction defect in cardiac muscle cells, and cataracts. Inheritance of DM is autosomal dominant (that is, a mutation in one copy of the affected gene causes diseases). There is also evidence of a tendency for maternal transmission of a severe congenital form of the disease. An unstable repeat sequence of triplet nucleotides (CTG) at the DM1 locus on chromosome 19q13.3 is the cause of DM1 in the majority of families [1,5]. The number of triplet repeats can increase with each generation, and disease severity correlates with the size of the expansion providing a molecular basis for anticipation. Unlike many other triplet diseases such as Huntington's disease where the repeats are in the coding region of the affected gene and results in an altered protein product, the CTG expansion at the DM1 locus is in the 3' non-coding region of the DMPK gene (which encodes a serine-threonine protein kinase) [6].

To explain these phenotypes shown in DM patient, we previously established cell model system for DM, i.e., C2C12 myogenic cells stably transfected with DMPK cDNA containing 160 CTG repeats in 3'untranslated

region [12]. We reported that DM model cell was highly sensitive against methyl-mercury induced oxidative stress [13]. These data lend support the hypothesis that the long CTG repeat in genome or long CUG RNA repeat may cause oxidative injury by regulating cellular antioxidant defenses. So in this study, we tried to measure the expression of antioxidant enzymes when cells were exposed to oxidants.

Materials and Methods

Cell Culture

Mouse myogenic clone C2C12 cells were maintained as described elsewhere [11,12]. Cells were co-transfected with pSRD-DMPK cDNA or pSV2-neo containing gene using the Lipofectamine Reagent (GIBCO BRL). DMPK cDNAs with CTG numbers of 5 or 130 were transfected. Stable transfectants were selected as described previously [12]. We analyzed RNA expression with respective CUG repeats by RT-PCR, although 130 CTG repeat have changed to 160 after experiment [12].

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Cell Viability Assay

To assay the susceptibility of cultured cells to oxidative stress, we used the trypan blue assay. First, myoblasts were plated in DMEM with 10% FBS into 24 well plates at a density of 7.5×10^4 cells/well. After 1 day, chemical oxidants were added to each well and the cell was observed for 24h. Next, living and dead cells were collected, and were dyed with trypan blue. Finally, the numbers of living and dead cells were counted with microscope.

Northern Blot Analysis

Total RNA was extracted from cultured cells by the standard acid guanidium-phenol-chloroform (AGPC) method. For Northern blot analysis, total RNA was separated by electrophoresis through a 1% agarose/formaldehyde gel blotted onto a nylon membrane and hybridized with ^{32}P -labeled cDNA probes. All cDNA fragments were isolated by PCR from human neonatal skeletal muscle cDNA library (clontech) using primers amplifying a part of open-reading-frame of each genes. Each fragment were obtained by digesting the plasmid pUC118 with EcoRI and XbaI

Results

High sensitivity to oxidative stress of DM model cell having long CTG repeat

In our initial investigation to measure sensitivity of DM model cells toward oxidants, we used hydrogen peroxides (H_2O_2) or menadione as a reagent. We counted living and dead cells by using trypan blue dye after exposure to antioxidant for 18 hour. The result is shown in Fig.1. Only C2C12 with long CTG repeats has high sensitivity to both oxidants. The largest difference is observed at 0.5mM H_2O_2 or 20 μM menadione, and at

those concentration, about 70% of the neo- or DMPK/5CTG-transfected cells were alive, while almost all of DMPK/160CTG-transfected cells were dead. These results indicate that mouse myogenic C2C12 cells with long CTG repeats may have lost scavenging system for superoxides.

Abnormal expression of antioxidant genes in DM model cell having long CTG repeat

To know the reason of high sensitivity of C2C12/DMPK/160CTG against oxidative stress, first, we presumed that the phenotype seen in mutant C2C12 cells can be caused by abnormal expression of antioxidant genes. Therefore we measured RNA expression by Northern blot analysis. Alpha-B-crystallin (CRYAB), copper/zinc superoxide dismutase (CuZn-SOD), mangano-superoxide dismutase (Mn-SOD) and glutathione peroxidase (GPx) were selected as typical antioxidants. CRYAB is known as a chaperon protein, and the others work as scavenger for superoxides. Total RNA was extracted from cells after exposure to oxidants at respective concentration shown in Fig.2. Each RNA sample extracted from C2C12/DMPK/5CTG cells or C2C12/DMPK/160CTG cells was transferred to nylon membrane, after separation by electrophoresis through a 1% agarose/formaldehyde gel. The hybridization signals were quantified after normalization to GAPDH signals. Each value was divided by that of 5CTG.

Decreased expression of alpha-B-crystallin was observed after oxidant treatment, but other enzymes (SODs and GPx) did not changed at all.

Discussion

We previously reported that DM model cell was highly sensitive against methylmercury-induced oxidative

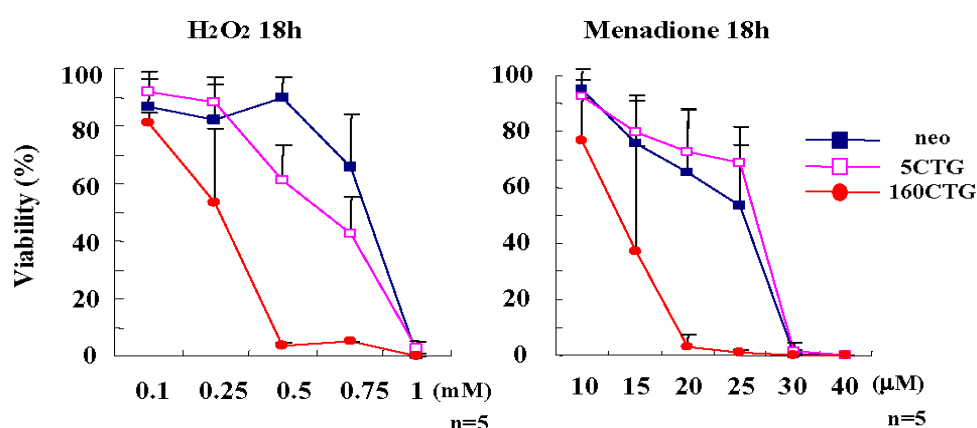


Fig.1. Effects of oxidizing reagents on cell viability in C2C12 cells. Viability was determined 18h after cell exposure to hydrogen peroxide (left) or menadione (right). The viability of untreated cells was regarded as 100%. Values represent means $\pm$ SD (n=5). Closed square, C2C12/neo cells; open square, C2C12/DMPK/5CTG cells; circle, C2C12/DMPK/160CTG cells.

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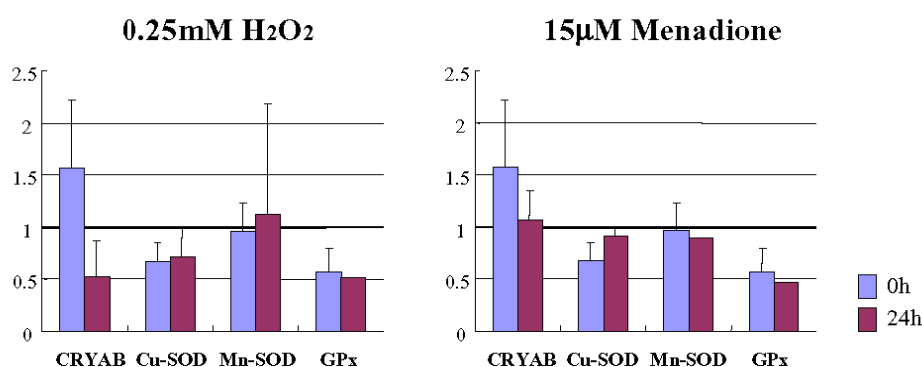


Fig.2. The ratio of mRNA expression to C2C12/DMPK/5CTG of C2C12/DMPK/160CTG. Expression was determined by Northern blotting after exposure to respective oxidants. The hybridization was quantified after normalization to GAPDH signals.

stress [11]. In this study, we used popular oxidizing reagents such as hydrogen peroxide and menadione, because influences of these oxidative stresses were already analyzed in dystrophin-deficient mouse models [2,8]. It was reported that muscle cells derived from mdx mouse were more sensitive against oxidative stress than the normal muscle cell, but the susceptibility to menadione between mdx and cells containing DMPK/160CTG was different. There was no difference in susceptibility to H₂O₂, but about 80% of the mdx mouse cells died at 5μM menadione, while few DMPK/160 CTG cells died at the same concentration. These oxidants are thought to act via different pathways, so we think that the different susceptibility between H₂O₂ and menadione is due to the difference in pathology between DM caused by long CTG repeat and Duchenne muscular dystrophy caused by the deficient of dystrophin protein.

Next, total RNA was isolated from these cell cultures and Northern blot analysis was performed. We used antioxidant genes like glutathione peroxidase or SODs as probes, because we suspected that the change in the expression of these enzymes could affect the susceptibility to oxidative stress of mutant cells. Another reason for carrying out antioxidant research is that previous report indicated the relation between antioxidant enzyme gene expression in response to oxidative stress and differentiation of mouse skeletal muscle [4]. We already reported that our model cells showed abnormal muscle differentiation [12]. So, we hypothesized that high sensitivity to oxidative stress in C2C12/DMPK/160CTG is caused by the change in antioxidant genes expression.

We observed the decrease in alpha-B-crystallin expression in C2C12 with longer CTG repeats. Therefore high sensitivity to oxidative stress in C2C12/DMPK/160CTG may be caused by an unusual expression of chaperon genes. Alternatively, it is highly possible that the sensitivity to oxidative stress could be caused by the activation of cell death or apoptosis

pathway. Recently RNA binding proteins such as triplet repeat expansion RNA-binding protein (EXP) and CUG repeat RNA-binding protein (CUG-BP) are said to be direct downstream for expansion RNA repeat and these protein's expression are changed in DM patients [7,9,10]. Therefore, it is possible that abnormal expression of alpha-B-crystallin was originated from abnormal behavior of these RNA binding proteins. By unraveling the mysteries of repeat-induced apoptosis, we will be able to provide hope for therapy of this disease.

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