Original Article

Localization of caspase 12 in masseter muscle of *mdx* mice during regeneration

Shinichi Abe

Oral Health Science Center HRC7 and Department of Anatomy, Tokyo Dental College

Abstract

The *mdx* mouse, a model of muscular dystrophy, lacks dystrophin, a cell membrane protein. It is known that this lack of dystrophin results in muscle fiber necrosis from 2 weeks after birth, and the majority of necrotic fibers are replaced by regenerated fibers by 4 weeks of age. Recent studies reported the detection of mitochondrial and endoplasmic reticulum stress proteins during muscle fiber necrosis in *mdx* mice, but did not histologically localize them to determine the timing of their expression during the process from cell necrosis to regeneration. Therefore, in this study, we investigated histological localization and gene-level expression in the *mdx* mouse masseter muscle of caspase-12 protein (among the caspases, which are cell stress-related genes) involved in the endoplasmic reticulum stress pathway. We observed caspase-12 expression in muscle cells that seemed to be in the process of necrosis in the *mdx* mouse masseter muscle at 2 weeks after birth, but not in regenerated muscle cells with centrally located nuclei observed at 3 to 4 weeks of age. These results suggest that due to the lack of dystrophin, it becomes difficult for muscle cells to maintain their morphology, and endoplasmic reticulum stress occurs to maintain cell morphology during the process of cell necrosis.

KEY WORDS: muscle anti-aging, regeneration, muscle fiber, masseter

Introduction

Duchenne muscular dystrophy (DMD) is caused by a lack of dystrophin in the subsarcolemmal cytoskeleton¹⁾. The *mdx* mouse, a model of muscular dystrophy, arose from a spontaneous mutation in inbred C57BL/10 mice, and was discovered in 1984 by Bulfield et al²⁾. Like DMD patients, *mdx* mice lack dystrophin, which results in muscle fiber necrosis and regeneration from 2 weeks after birth, and the majority of necrotic muscles of the extremities are replaced with regenerated muscles by 4 weeks of age³⁻⁵⁾. A similar phenomenon was reported to occur in the branchial arch-derived masseter muscle of *mdx* mice⁶⁾. Thus, *mdx* mice have been used as the optimal model for studying the pathogenesis of muscle necrosis⁷⁾.

In recent years, an apoptosis-like phenomenon that occurs during necrosis has been discussed^{8,9)}. In regard to this, a study investigating overexpression of the caspase-3 gene related to mitochondrial-mediated apoptosis has noted that, in addition to necrosis, a phenomenon similar to apoptosis may occur in the muscle fibers of the *mdx* mouse model of muscular dystrophy¹⁰⁾. Another study has reported the occurrence of not only mitochondrial- but also endoplasmic reticulum-mediated intracellular stress during muscle fiber necrosis in *mdx* mice¹¹⁾. These studies have shown that cellular necrosis occurred via intracellular stress, as demonstrated for apoptosis, even in the process of necrosis; however, no study has investigated the tissue localization of caspases. In this study, we examined the localization of caspase 12 in the masticatory muscle of *mdx* mice using an immunohistochemical method.

Material and Methods

Specimens

Three groups of 10 *mdx* male mice (C57BL/10ScSn) at the ages of 2, 3, 4 weeks, respectively, and groups of 10 control mice (B10 Scott Snells) at the corresponding ages were used in the study. The mice were anesthetized with pentobarbital, and sacrificed according to the Guidelines for Animal Experiments of Tokyo Dental College. The superficial layers of the left masseter muscle from all mice were used for morphological examination and immunohistochemical analysis.

Frozen sections

The masseter muscle tissue was embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), placed in isopentane (-160°C) and refrigerated with liquid nitrogen to quicken the freezing process. Subsequently, according to the conventional method, serial fresh-frozen sections of 8 µm, perpendicular to the grain of muscle fibers, were prepared at -20°C in a cryostat. Some frozen sections were stained with hematoxylin eosin (HE) after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4.

Antibody

The following antibody was used in this study: Polyclonal Rabbit Anti-Active Caspase-12: 3015-100 (Bio Vision Research Products, CA, USA), dilution 1:500, 4°C overnight.

Immunohistochemistry

Immunohistochemistry was performed using the Vectastain Elite ABC Kit, Rabbit IgG (Vector Laboratories, Inc., USA) according to the manufacturer's instructions. Some frozen sections were transferred into PBS after fixation in 4% paraformaldehyde in PBS for 5 minutes. To quench endogenous peroxidase, the sections were treated with 0.3% H₂O₂ in methanol for 30 minutes, followed by rinsing with PBS. Sections were next treated with 0.1% goat normal serum in PBS at room temperature for 30 minutes and reacted with the above primary antibody diluted in PBS. The sections were treated with biotinylated secondary anti-rabbit antibody for 30 minutes, followed by ABC reagent for 30 minutes at room temperature. Next, they were incubated with peroxidase substrate solution (DAB Solution, Wako Chemicals, USA, Inc.) at room temperature for 3 minutes, and then transferred into distilled water. Finally, they were stained with methyl green for counterstaining, dehydrated in ethanol, and mounted with Pristine Mount (Falma, Inc., Japan).

Reverse transcription polymerase chain reaction analysis

From mice in each age group, the muscle was removed and snap-frozen in liquid nitrogen. mRNA at each stage was extracted using a QuickPrep micro mRNA Purification Kit (Amersham Pharmacia Biotech UK Ltd.), and cDNA was prepared using Ready-To-Go (Amersham Pharmacia Biotech UK Ltd.). After the optimal PCR conditions for all primers were determined, experiments were performed using a LightCycler™ (Roche Diagnostics, Mannheim, Germany), which allows RNA quantification. Experiments were performed according to the standard protocol for the LightCycler™. A ready-to-use LC FastStart DNA Master SYBR Green I (Roche) was used as a hot-start PCR reaction mix for the LightCycler™. A series of dilutions of a cDNA synthesis (4.0 ng/μl) were made, and dilution ratios of 1/10⁵, 1/10⁶, 1/10⁷, 1/10⁸, and 1/10⁹ were used. The PCR product contained 10.2 μl of sterile water and 5 μl of diluted control cDNA, 1.6 μl of 25 mM MgCl₂, and 2 μl LC FastStart DNA Master SYBR Green I containing SYBR Green I (1/60,000

dilution). In addition, 0.6 μl of each forward and reverse primer were added to a final reaction volume of 20 μl. The mRNA sequence of the caspase-12 obtained from the genome database was used to design specific primers (Biogene Ltd.), and had the following sequences: caspase-12 (forward, 5'-AGGATGATGGACCTCAGA-3'; reverse, 5'-GCTGTCAGCATTAGATGTGA-3'; Accession No. NM_009808). PCR mixtures (20 μl each) prepared for caspase-12 were added to the glass portion of a capillary. Initial denaturation at 95°C for 10 min was followed by 45 cycles of denaturation at 95°C for 10 sec, an annealing step at 60°C for 10 sec, and an extension step at 72°C for 8 sec. Gene amplification was performed according to a melting program of 70°C for 15 sec, and fluorescence was continuously monitored at a rate of 0.1 deg/s from 70 to 95 deg. Fluorescence channel F1 (530 nm) was used, and the gain volumes for caspase-12 were 82.6°C, 89.6°C, 91.4°C and 82.6°C, respectively.

The numerical values of each caspase-12 mRNA expression were normalized by dividing the expression of housekeeping GAPDH mRNA to finally obtain the relative expression level of mRNA. The primers for GAPDH had the following sequences: forward, 5'-TGA ACGGGAAGCTCACTGG-3'; reverse, 5'-TCCACCACCCTGTTGCTGTA-3'; Accession: NM_008084.

Statistical analysis

Student's t-test was applied to pairs of each time-point values in this study and a p value of <0.05 was considered significant.

Results

Morphological observations

Features suggestive of muscle cell necrosis were noted in the masseter muscle of 2-week-old *mdx* in comparison with control mice. In addition, a small number of regenerated muscle fibers with centrally located nuclei were seen in the masseter muscle of 3-week-old *mdx* mice, and a larger number of regenerated muscle fibers in that of 4-week-old *mdx* mice (**Figure 1**).

Fig. 1 Representative histological observations from *mdx* and control mice (B10) at each time point. Scale bars = 100 μm, H&E staining. N: necrotic cell; R: regenerated cell

Immunohistochemical staining results

Some muscle cells of 2-week-old *mdx* mice were positively stained with anti-caspase-12 antibody. Anti-caspase 12 antibody-positive cells were smaller than peripheral cells and had an irregular shape. These cells were somewhat different in morphology from the surrounding cells, and it was suggested that they were in the early stage of necrosis. In the masseter muscle of 3- and 4-week-old *mdx* mice, many regenerated muscle fibers with centrally located nuclei were observed, but no areas were positively stained with anti-caspase-12 antibody (Figure 2).

In the masseter muscle of control B10 mice at various weeks of age, no areas were positively stained with anti-caspase-12 antibody (Figure 2).

Expression of caspase-12 mRNA

The average expression levels of each caspase-12 mRNA from 10 *mdx* mice and 10 control mice at each time point are shown in Figure 3. The level of caspase-12 mRNA expression was significantly higher in 2-week-old *mdx* mice than in control mice, but did not significantly differ between 3- and 4-week-old *mdx* and control mice ($p < 0.05$).

Fig. 2 Immunohistochemical staining results. Anti-caspase 12 antibody-positive cells were observed in the masticatory muscle of 2-week-old *mdx* mice (arrow).

Fig. 3. Average expression level of each caspase-12 mRNA from *mdx* mice and control mice (B10) at each time point (LightCycler™, Values are the mean ± SD). Ordinate shows relative gene expression unit as compared to the control housekeeping GAPDH gene. Caspase 12 mRNA expression in the *mdx* mouse masticatory muscle at 2 weeks of age was significantly higher than that at 3 or 4 weeks of age; however, there were no age-related differences in control mice.

Discussion

In recent years, it has been reported that intracellular stress generated during myogenesis plays an important role in selecting stress-resistant cells and killing stress-sensitive cells¹²⁾. In multidisciplinary studies connected with regenerative medicine, inhibitory factors during myogenesis have attracted attention.

Cell death is classified into necrosis according to morphological and biochemical characteristics¹³⁾. Necrosis is caused by major environmental changes deviating from cell homeostasis, such as ischemia and injury. In contrast, necrosis is closely regulated by intracellular signals, and is not associated with cell membrane disruption, causing little inflammatory reaction; therefore, it has conventionally been considered that physiological cell death proceeds by apoptosis and pathological cell death by necrosis.

The activation cascade of proteases called caspases plays an important role in the execution of apoptosis^{14,15)}. Endoplasmic reticulum- and mitochondrial-mediated pathways are known to be involved in their activation. The involvement of endoplasmic reticulum stress-induced apoptosis in the activation of caspase-12 has been suggested¹⁶⁾.

Mitochondrial-mediated pathways have been reported to be involved in the activation of Bax, caspase-9, and caspase-3¹⁷⁾. Recently, it has been noted that, in addition to necrosis, a phenomenon similar to apoptosis may occur in the muscle fibers of the *mdx* mouse model of muscular dystrophy. A recent study, investigating overexpression of the caspase-3 gene related to mitochondrial-mediated necrosis, noted that, in addition to necrosis, a phenomenon similar to apoptosis may occur in the muscle fibers of the *mdx* mouse model of muscular dystrophy¹⁰⁾. In addition, the study analyzed the expression of caspase-3, -9, -12, and Bax during muscle necrosis due to the lack of dystrophin, and investigated mitochondrial and endoplasmic reticulum stress; however, their histological localization has not been ascertained. Therefore, in this study, we immunohistochemically localized the expression of caspase-12, which is known to be involved in endoplasmic reticulum stress, during necrosis of the *mdx* mouse masseter muscle.

We found that, in the *mdx* mouse masseter muscle, caspase-12 was expressed in cells showing a morphology somewhat different from that of the surrounding cells. The level of caspase-12 mRNA expression was also significantly higher in 2-week-old than in control mice. Since caspase-12 is considered to be a necrosis-related protein induced by endoplasmic reticulum stress¹⁶⁾, the results of this study suggest that the lack of dystrophin leads to the generation of stress in muscle fibers to maintain their structure. The reduced caspase-12 expression in the masseter muscle of 3- and 4-week-old *mdx* mice suggested that cell necrosis had already started, and endoplasmic reticulum stress had disappeared. In this study, caspase 12 expression was detected in the masticatory muscle of *mdx* mice, as previously reported. Concerning tissue localization, which has been unclear, the results suggest that caspase 12 appears in the initial phase of cellular necrosis.

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