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Author(s) Alternative	Honda, A; Abe, S; Hiroki, E; Honda, H; Iwanuma, O; Yanagisawa, N; Ide, Y
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Activation of caspase 3, 9, 12 and Bax in masseter muscle of mdx mice during necrosis

A. Honda[1], S. Abe[1, 2 *], E. Hiroki[1], H. Honda[1], O. Iwanuma[1], N. Yanagisawa[3], Y. Ide[1]

[1]*Department of Anatomy, Tokyo Dental College 1-2-2 Masago, Mihama-ku, Chiba-City 261-8502, Japan*

[2]*Oral Health Science Center HRC7, Tokyo Dental College 1-2-2 Masago, Mihama-ku, Chiba-City 261-8502, Japan*

[3]*Department of Anatomy Showa University School of Dentistry, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan.*

[*] To whom correspondence should be addressed. Phone: +81-43-270-3571; Fax: +81-43-277-4010; E-mail: abesh@tdc.ac.jp

Abstract

The mdx mouse, a model of muscular dystrophy, lacks dystrophin, a cell membrane protein. It is known that the lack of dystrophin causes muscle fiber necrosis from 2 weeks after birth, and the majority of necrotic muscle fibers are replaced by regenerated muscle fibers by 4 weeks after birth. A recent study indicated the possibility that mitochondria-mediated intracellular stress, a phenomenon similar to apoptosis, may be produced during muscle fiber necrosis, but did not analyze endoplasmic reticulum-mediated intracellular stress. Therefore, we examined the expression of the caspase-12 gene involved in the endoplasmic reticulum stress pathway and the Bax, caspase-9, and caspase-3 genes involved in the mitochondrial stress pathway in the mdx masseter muscle. We found over-expression of caspase-12 in cells at 2-3 weeks after birth when muscle fiber necrosis was not prominent. This suggests that stress occurs in the endoplasmic reticulum to maintain cell morphology in the absence of dystrophin. In addition, Bax was abundantly expressed in the mdx masseter muscle at 3 weeks after birth, and the expression of caspase-9 and -3 was prominent at 3-4 weeks after birth when necrosis and regeneration were marked. These results indicate that endoplasmic reticulum and mitochondrial stresses are produced during necrosis of

the mdx masseter muscle, and suggest that these events are a phenomenon similar to apoptosis.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle disorder caused by the lack of dystrophin, a 427-kDa protein located in the subsarcolemmal space of muscle fibers (Hoffman et al. 1987). The mdx mouse, a model of DMD, resulting from a mutation in the dystrophin gene in a C57BL/10 mouse, was discovered by Bulfield et al. (1984). Similar to DMD patients, the mdx mouse lacks dystrophin. It was reported that, in somite-derived muscles of the extremities, muscle necrosis began at 2 weeks after birth, immediately followed by regeneration, and the majority of necrotic muscles became regenerated at 4 weeks after birth (Dangain et al. 1984; DiMario et al. 1991; Attal et al. 2000). A similar phenomenon was noted in the branchial arch-derived mdx mouse masseter muscle (Lee et al. 2006), and cell degeneration in this muscular dystrophy was ascribed to the lack of dystrophin, resulting in damage to cell membrane stability, leading to excessive influx of calcium ions into muscle cells, a mechanism similar to that of necrosis. Thus, mdx mice have been used as a model to study the mechanism of muscle

necrosis (Gillis et al. 1996).

An apoptosis-like phenomenon that occurs during necrosis has been discussed in recent years (Graeber et al. 2002; Mattson 2000). A related study indicated that, in addition to necrosis, an apoptosis-like phenomenon occurs in the muscle fibers of the mdx mouse model of muscular dystrophy. That study analyzed over-expression of caspase-3, which is related to mitochondrial-mediated apoptosis (Mizutani et al. 2005), but not in further detail.

In this study, to clarify the expression of apoptosis-related caspase genes during necrosis of muscle cells lacking the membrane protein dystrophin, we examined the mdx mouse masseter muscle for caspase-12 protein involved in the endoplasmic reticulum stress pathway, and Bax, caspase-9, and caspase-3 proteins involved in the mitochondrial stress pathway.

Materials and methods

Muscle biopsy Specimen

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. The right masseter muscle from all mice was used for examination at the protein and the transcriptional levels.

Morphological examinations

The masseter muscle was frozen immediately after removal and mounted on a cork block. 7μm-thick serial cryostat sections were prepared at right angles to the longitudinal axis of muscle fibers, stained with H & E, and examined under a universal photomicroscope (UPM Axiophot2).

Western blotting analysis of caspase-12, Bax, caspase-9 and caspase-3

Electrophoretic separation and analysis of the protein bands by western blotting was performed. Briefly, the masseter muscles from 2, 3 and 4 weeks of mdx and control mice were removed while the animals were anaesthetized. The muscles were weighed, frozen in liquid nitrogen, and stored at -80℃. Frozen muscles were minced with scissors in 9 volumes of ice-cold homogenization buffer (250 mM sucrose, 100 mM KCl, 5 mM ethylenediaminetetraacetic acid, and 20 mM Tris hydroxymethyl

aminomethane (Tris), pH 6.8). The minced muscle samples were subsequently sonicated in a Branson Sonifier 250D (Branson Ultrasonic Corporation, Danbury, CT, USA). The products were used for the preparation of washed myofibers, which were then boiled in sample buffer for 2 min at a final protein concentration of 0.125 mg/ml. Total proteins were determined by the Bradford technique using the Bio-Rad Protein Assay (Nippon Bio-Rad Laboatory) and a Gene Quantpro spectrophotometer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Western blotting analyses were performed for the detection of caspase-3, -9 and -12 and Bax. Equal amount of total protein for each group (40 μg) was separated on 12.5% SDS-polyacrylamide gel and was transferred to Immobilon P membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk and incubated with each of primary antibodies of anti-Caspase-12 (1:1000, BioVision 3282-100), anti-Bax (1:500,Aviva Systems Biology AVARP02020 T200), anti-Caspase-9 (1:1000, BioVision 3409-100), and anti-Caspase-3 (1:1000, BioVision 3138-100) antibody, and detected with horseradish peroxidase-conjugated secondary anti-rabbit/mouse IgG antibody using the ECL plus system (GE Healthcare, Buckinghamshire, UK).

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 $1/10^5$, $1/10^6$, $1/10^7$, $1/10^8$, and $1/10^9$ 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 $MgCl_2$, 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 of forward and reverse primers were added to a final reaction volume of 20 µl. The mRNA sequences of the caspase-12, Bax, caspase-9 and caspase-3 genes obtained from genome database were used for the design of specific primers (Biogene Ltd.) for each gene, and had the following sequences: caspase-12 (forward,

5'-AGGATGATGGACCTCAGA-3'; reverse, 5'-GCTGTCAGCATTAGATGTGA-3'; Accession No. NM_009808), Bax (forward, 5'-GAAGGCCTCCTCTCCTACC-3'; reverse, 5'-AAGACACAGTCCAAGGCA-3' Accession No. NM_007527), caspase-9 (forward, 5'-ATGGTCACGCTTTGATGGA-3'; reverse, 5'-TTCTCAATGGACACGGA GCA-3'; Accession No. NM_015733), caspase-3 (forward, 5'-GACCATACATGGGAGCAAGT-3'; reverse, 5'-ATCCGTACCAGAGCGAGA-3'; Accession No. AK0 14231). The PCR mixtures (20 μl each) prepared for caspase-12, Bax, caspase-9, caspase-3 were added to the glass portion of capillary. Initial denaturation at 95℃ for 10 min was followed by 45 cycles of a denaturation step at 95℃ for 10 sec, an annealing step at 60℃ for 10 sec, and an extension step at 72℃ for 8 sec. Gene amplification was performed according to a melting program of 70℃ 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, Bax, caspase-9 and caspase-3 were 82.6℃, 89.6℃, 91.4℃ and 82.6℃, respectively.

The numerical values of each of caspase-12, Bax, caspase-9 and caspase-3 mRNA expressions were divided by 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'; Ac- cession: 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

Histological staining of masseter muscle from mdx and control B10 mice is shown in figure 1. At the age of 2 weeks, the histological features were not different between mdx mice and control mice. However, degeneration of muscle fibers was identified in the 3-week-old mdx mice. On the other hand, both degenerated and regenerated muscle fibers with central nuclei were revealed in the 4-week-old mdx mice.

Western blotting analysis of caspase-12, Bax, caspase-9 and caspase-3

Electrophoretic data from the masseter muscle of mdx and control mice are illustrated in Figure 2. Analysis of protein expression revealed that caspase-12,

which was hardly detected in the control mice, was strongly expressed in mdx mice particularly at the age of 2 and 3 weeks. Bax, which was hardly expressed in the control mice, was strongly expressed in mdx mice especially at the age of 3 weeks. Caspase-3 and -9, which were hardly detected in the control mice, were strongly expressed in mdx mice particularly at the age of 3 and 4 weeks.

Expression of caspase-12, Bax, caspase-9 and caspase-3

The average expression level of each of caspase-12, Bax, caspase-9 and caspase-3 mRNA from 10 mdx mice and 10 control mice at each time point is shown in Figure 3. The level of caspase-12 mRNA expression was significantly higher in 2- and 3-week-old mdx than in control mice, but did not significantly differ between 4-week-old mdx and control mice. The level of Bax mRNA expression was significantly higher in 3-week-old mdx than in control mice, and slightly but not significantly higher in 4-week-old mdx than in control mice. The expression levels of caspase-9 and caspase-3 did not significantly differ between 2-week-old mdx and control mice, but were significantly higher in 3- and 4-week old mdx than in control mice ($p<0.05$).

Discussion

Cell death is classified into necrosis and apoptosis according to morphological and biochemical characteristics (Fink et al. 2005). Necrosis is caused by large environmental deviations from cellular homeostasis, such as ischemia or injury, and is associated with disruption of the cell membrane. In contrast, apoptosis is regulated by intracellular signals, is not associated with disruption of the cell membrane, and induces little inflammation. Therefore, physiological cell death in organogenesis and pathological cell death had been considered to proceed by apoptosis and necrosis, respectively. This simple view is recently questioned since the involvement of apoptosis in neurodegenerative diseases was demonstrated, and apoptosis occurring during necrosis came to be discussed (Graeber et al. 2002; Mattson 2000). However, there are few such studies, and the issue has not been clarified.

The activation of the caspase cascade plays a central role in the execution of apoptosis (Friedlander et al. 2003; Shi el al. 2002), and is known to involve the signaling pathway mediated by the cell-membrane death receptor, endoplasmic reticulum, and mitochondrion. Caspase-12 was shown to mediate endoplasmic reticulum stress-induced apoptosis (Nakagawa et al. 2000). In the

mitochondria-mediated signal pathway, the apoptosis-promoting factor Bax in the cytoplasm migrates to mitochondria, and releases cytochrome c from mitochondria. The released cytochrome c binds to Apaf-1, resulting in the activation of caspase-9. The activated caspase-9 has been reported to activate downstream caspase-3, thereby executing apoptosis (Earnshaw et al. 1999). A recent study analyzed over-expression of caspase-3, and indicated that not only necrosis but also an apoptosis-like phenomenon occurred in muscle fibers in the muscular dystrophy (mdx) mouse model (Mizutani et al. 2005). However, that study did not examine whether endoplasmic reticulum-mediated intracellular stress occurred during necrosis due to the lack of dystrophin.

The results of this study showed over-expression of caspase-12 in the masseter muscle of mdx mice at 2-3 weeks after birth, when the cell morphology was nearly normal, with no evidence of necrosis. Caspase-12, localized on the cytoplasmic side of the endoplasmic reticulum, has been reported to be an apoptosis-related protein induced by endoplasmic reticulum stress (Nakagawa et al. 2000). Therefore, the results of this study suggest that the lack of dystrophin protein results in excessive stress in muscle fibers to maintain their structure. In addition, the decreased expression of caspase-12 in the masseter muscle of 4-week-old mdx mice suggests

that cell necrosis began, and the endoplasmic reticulum stress disappeared. In contrast, the prominent expression of caspase-9 and caspase-3 in the masseter muscle of mdx mice at 3-4 weeks after birth, just after the expression of Bax, suggests that mitochondria-mediated intracellular stress occurs during cell disintegration. A recent study of myogenesis has reported that during the process of differentiation of myoblasts into myotubes, mitochondria-mediated intracellular stress occurs, and selectively disposes of cells that are susceptible to cellular stress. In conclusion, although ER stress occurs at the beginning, it does not have a major physiological function. Thus cell destruction starts for the first time in the mitochondria-dependent pathway occurring at 3-4 weeks. Moreover, mitochondria-dependent apoptosis happens in some of the cells at the regenerative stage.

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Figure Legends

Figure 1 Representative histological observations from mdx and the control mice at each time point. No significant difference was detected between mdx mice and the control mice in 2 weeks of age. Muscle fiber necrosis was revealed in 3-week-old mdx mice. Both degenerated and regenerated muscle fibers with central nuclei were observed in 4-week-old mdx mice (Scale bars=100 μm, H&E staining).

Figure 2. Protein expressions of Caspase-12, Bax, caspase-9 and caspase-3 of mdx and control mice determined by electrophoresis. Lane 1, 2-week-old; lane 2, 3-week-old; lane 3, 4-week-old for each of mdx and the control mice.

Figure 3. Average expression level of each of caspase-12, Bax, caspase-9 and caspase-3 mRNA from 10 mdx mice and 10 control mice at each time point

(LightCyclerTM, Values are mean±SD). Ordinate shows relative gene expression unit as compared to the control housekeeping GAPDH gene.

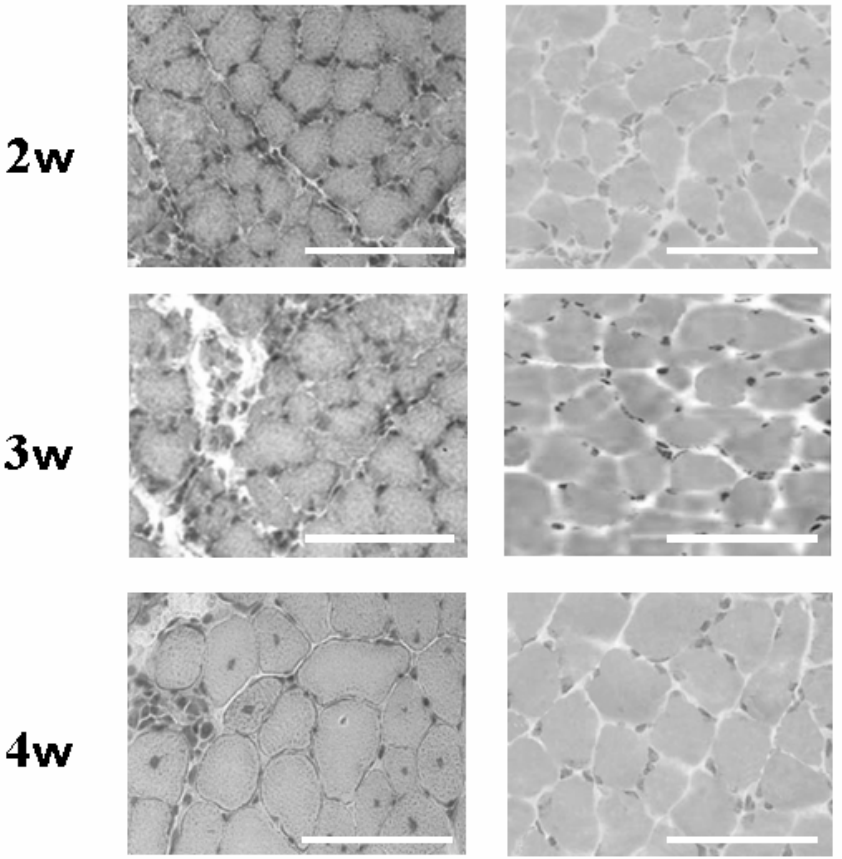


Fig.1

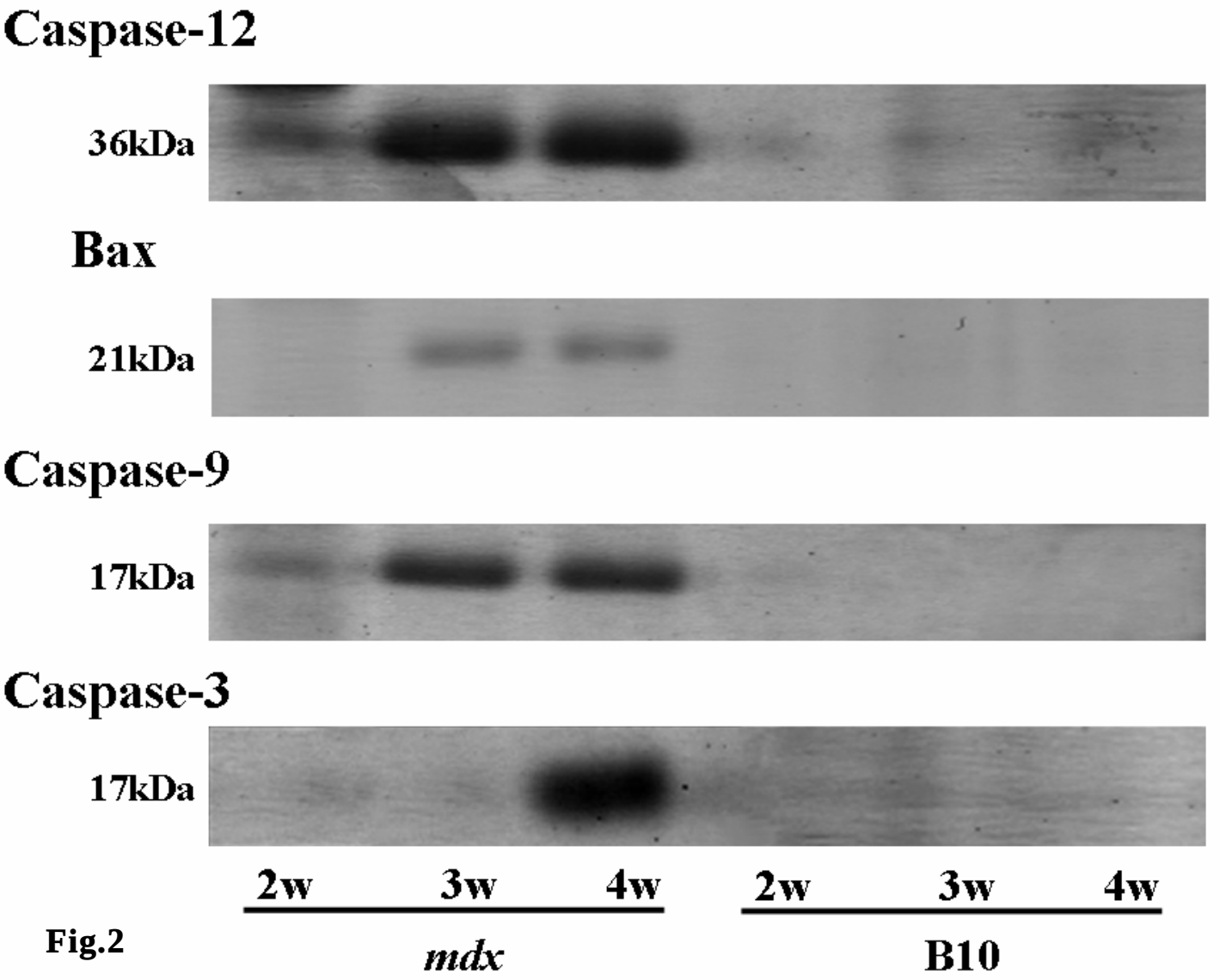
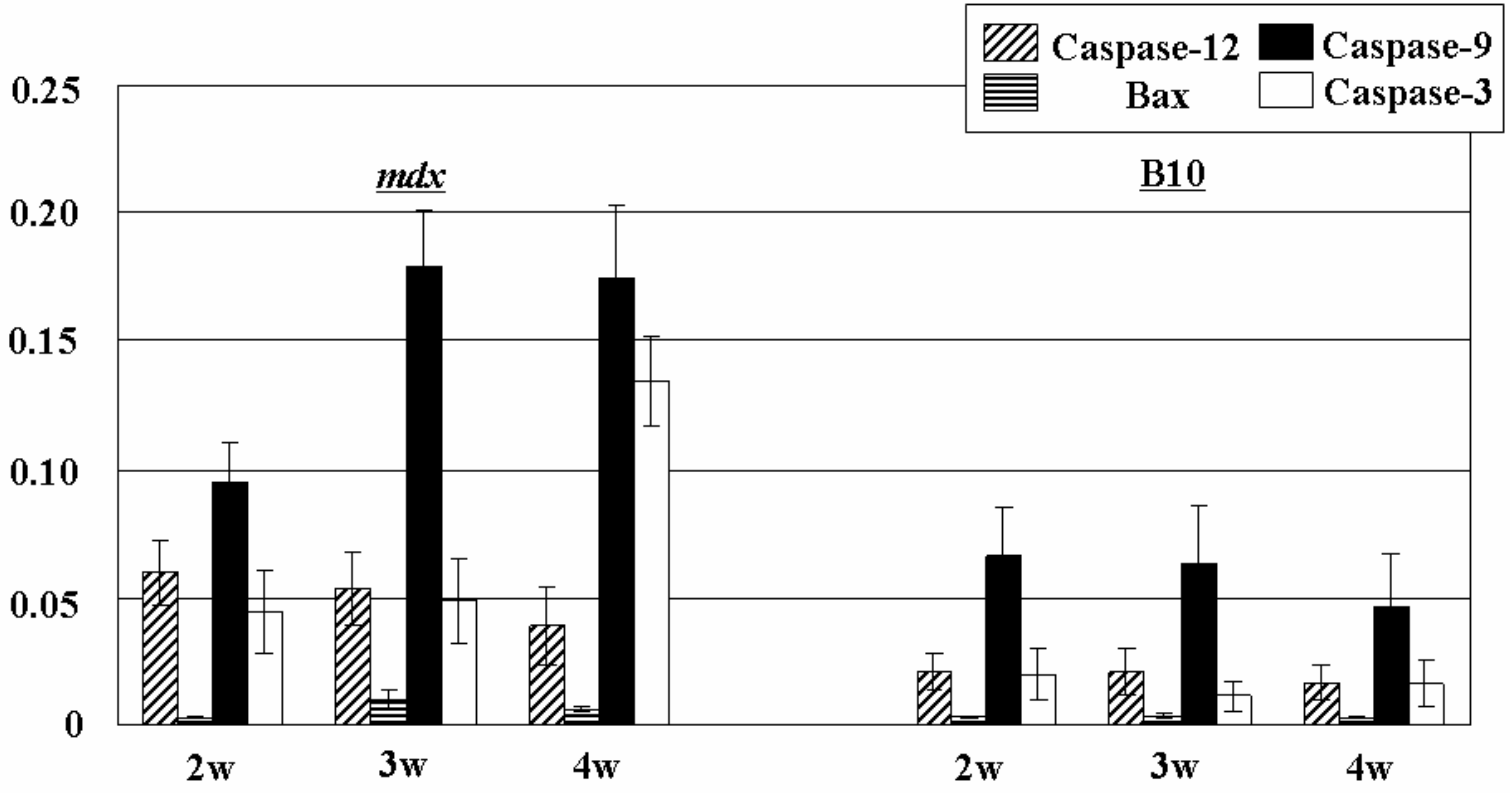

Caspase-12
36kDa
Bax
21kDa
Caspase-9
17kDa
Caspase-3
17kDa
2w
3w
4w
2w
3w
4w
mdx
B10

Fig.2



0.0595*	0.0538*	0.0389		0.0211*	0.0208*	0.0165
0.0029	0.0098*	0.0064*		0.0032	0.0034*	0.0029*
0.0953	0.1798*	0.1753*		0.0658	0.0635*	0.0463*
0.0443	0.0485*	0.1345*		0.0198	0.0115*	0.0165*

Fig.3