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Serum Calcium-decreasing Factor, Caldecrin, Ameliorates Muscular Dystrophy in *dy/dy* Mice

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Abstract. *Background: Calcium signaling is important in muscular cells and abnormal Ca^{2+} handling results in muscle damage. Caldecrin is a serum calcium-decreasing factor purified from pancreas. It is a chymotrypsin-type secretory protease, whereas the serum calcium-decreasing activity does not depend on its protease activity. Here, we evaluated the effect of caldecrin on dystrophia muscularis (dy/dy) mice. Materials and Methods: Caldecrin gene in hemagglutinating virus of Japan envelop vector (10 μg/head) was single injected in the femoral muscle or daily intraperitoneal administration of caldecrin (100 μg/kg body weight) was given for 4 days in dy/dy mice. Progression of muscular dystrophy was assessed by blood urea nitrogen and Evans blue dye penetration assay. Results: Ectopic expression of the caldecrin gene in the muscle of dy/dy mice reduced the level of blood urea nitrogen and improved the dystrophic progression similarly to that of caldecrin-injected mice. Conclusion: Caldecrin treatment is effective for preventing muscular dystrophy.*

Skeletal muscle damage and degeneration are caused by various factors, such as excessive exercise training, mechanical or pharmacological injury, ischemia, inflammation, and the genetic disease of muscular dystrophy (1). Absence of the membrane cytoskeletal protein dystrophin results in muscular dystrophy (2). Numerous studies using the genetic mouse model and pharmacological studies have provided the molecular basis of muscular dystrophy, although there is no effective cure for this disorder. In the muscle fiber, dystrophin and syntrophin, which bind to F-actin, form a complex with membrane glycoproteins, β-dystroglycan and α-sarcoglycan (3-4). Dystroglycan also binds to extracellular matrix laminin. This complex formation is important for the transduction of myosin movement toward the extracellular matrix. A defect of part(s) of this complex causes Duchenne (dystrophin deficiency), Becker (dystrophin mutation), limb girdle (LG: sarcoglycan mutation), and other muscular dystrophies (5-9).

Pathways involved in muscular damage, such as the loss of intracellular Ca^{2+} homeostasis, loss of energy supply to the cells, oxidative stress and activation of apoptosis, have been reported (10-12). Intracellular Ca^{2+} is elevated in Duchenne muscular dystrophy and its mouse model, the *mdx* mouse, leading to the activation of Ca^{2+}-dependent calpain proteolysis (13-14). Resting Ca^{2+} levels in the fibers from dystrophic *dy/dy* mice, in which the laminin α2 chain (15-17) is deficient, were elevated two- to four-fold compared to normal fibers (18). This evidence strongly suggests the implication of abnormal calcium handling in muscle disease.

In 1977, Takaoka *et al*. found that the anabolic factor(s) extracted from porcine pancreas, named Px (pancreas extracts), reversed and/or delayed the progression of fascioscapulohumeral muscular dystrophy (FSHD) and limb girdle muscular dystrophy (LGMD) in Japan (19). They noted that the activity of Px in improving dystrophic muscle was correlated with serum calcium- and blood urea nitrogen (BUN)-decreasing activity as evaluated by a bioassay using rabbit. BUN in serum reflects the degree of muscular degradation. The level of BUN increased in the patients with early muscular dystrophy (20-22). We purified caldecrin protein, as a serum calcium-decreasing factor, from porcine (23) pancreas and cloned the rat (24) and human (25) caldecrin gene. Caldecrin is a secretory-type serine protease which has chymotryptic activity and is also known as chymotrypsin C [EC3.4.21.2] (23-24). Caldecrin dose-dependently inhibited serum calcium concentration. Interestingly, this activity depends on its being processed to

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the mature form from the pro form, but not on its protease activity, because protease-deficient mutant caldecrin also possessed serum calcium-decreasing activity. We reported that caldecrin suppressed the bone-resorbing activity of rabbit mature osteoclasts (26). Recently, we found that caldecrin inhibits osteoclast differentiation by the suppression of nuclear factor of activated T-cell cytoplasmic 1 (NFATc1) activity (27). Caldecrin inhibits the receptor activator of nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) ligand (RANKL)-induced Ca^{2+} oscillation, which is an important cellular event for NFATc1 activation. Taking these findings together with the effects of Takaoka's Px on muscular dystrophy, we hypothesized that caldecrin may antagonize the Ca^{2+} elevation that results in pathogenesis of muscle cells.

In this study, we examine the therapeutic effects of caldecrin on muscular dystrophy.

Materials and Methods

Animals. Female ICR mice (7-8 weeks old) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Male muscular dystrophic mice (C57BL6J *dy/dy*) and littermates (5 weeks old) were obtained from the Central Institute for Experimental Animals (Kanagawa, Japan). Animals had free access to food and water. The animals were acclimatized for at least 1 week in the animal facilities of Meikai University. All experiments were performed according to Meikai University School of Dentistry Animal Care Guidelines.

Preparation of Flag-tagged-rat caldecrin expression vector and purification of recombinant rat caldecrin protein. Rat caldecrin cDNA was PCR amplified using pCAGGS-rat caldecrin cDNA (24) as a template and ligated in the pIRES-hrGFP-1a vector (Stratagene, Jolla, CA, USA). The expression vector of rat caldecrin (prCaldecrin3×Flag-IRES-hrGFP) was transfected into HEK293T cells (American Type Culture Collection) and recombinant rat caldecrin was purified by anti-Flag M2 agarose column (Sigma-Aldrich, St. Louis, MO, USA).

Preparation of human caldecrin expression vector and purification of human caldecrin protein. Human caldecrin gene isolation and purification of recombinant were described elsewhere (25, 28). Human caldecrin gene was PCR amplified from the cDNA library (Stratagene) and ligated in the pEXPR-IBA3 vector (IBA, Göttingen, Germany) (hCaldecrin-strep). Using the hCaldecrin-strep cDNA as a template, PCR was performed and ligated into pIRES-bleo3 vector (Clontech Laboratories Inc., Mountain View, CA, USA). The expression vector of human caldecrin (phCaldecrin-strep-IRES bleo) was transfected into HEK293T cells and selected with bleomycin (400 μg/ml) for 2 weeks. The culture medium containing secreted recombinant human caldecrin was precipitated with 65% ammonium sulfate and purified by Strep-Tactin immobilized gel (IBA) and Mono S ion-exchange chromatography (GE Healthcare Japan Co., Tokyo, Japan).

The recombinant caldecrin was freshly activated by treatment with trypsin (50:1) for 30 min at room temperature followed by treatment with 0.1 mM 4-amidinophenylmethanesulfonyl fluoride hydrochloride (Sigma-Aldrich) to terminate activation.

Preparation of hemagglutinating virus of Japan envelope vector (HVJ-E) for caldecrin gene. HVJ-E containing the prCaldecrin3× Flag-IRES-hrGFP vector or phCaldecrin-strep-IRES-bleo vector was prepared by using GenomOne-Neo (Ishihara Sangyo Kaisya, Ltd: Osaka, Japan) according to the manufacturer's protocol and contains the HVJ envelope for adhesion and delivers the gene into the cell (29).

In vivo treatment with caldecrin gene and recombinant caldecrin. For the gene expression experiments in mice, the femoral muscle of ICR mice (7-8 weeks old, 4 mice per group) was transfected with GenomOne-Neo containing prCaldecrin3×Flag-IRES-hrGFP cDNA (10 μg/100 μl per lateral muscle) or the same volume of the control vehicle by intramuscular injection under anesthetic. At different times (3, 7 and 14 days after injection), the injected muscle was dissected and serum was collected to determine the caldecrin gene expression.

For the dystrophic muscle gene therapy, GenomOne-Neo containing phCaldecrin-strep-IRES-bleo cDNA (10 μg/100 μl per lateral muscle) was injected into the lateral femoral muscle of 8-week-old muscular dystrophic *dy/dy* mice or littermates (3 mice per group). The mice were fasted overnight before being sacrificed on day 5 after injection.

For the dystrophic muscle therapy with protein administration, purified human caldecrin (100 μg/ kg body weight) or control buffered saline was daily intraperitoneally administered for 4 days; mice were then fasted overnight before the last administration and sacrificed 3 hours after the last injection.

ELISA for Flag-tagged caldecrin. To measure the levels of secreted Flag-tagged caldecrin in the blood, the sera were prepared 3, 7 and 14 days after gene transfection in the muscle as described above. The serum (200 μl) was applied to a 96-well microtiter plate coated with anti-FLAG HS, M2 antibody (Sigma-Aldrich) and incubated at 37˚C for 1 h. After washing, bound caldecrin was reacted with polyclonal anti-rat caldecrin antibody at room temperature for 1 h, and then detected with alkaline phosphatase-conjugated anti-rabbit IgG antibody for 1 h and then the substrate solution (250 μl) containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM *p*-nitrophenol phosphate was applied to the well and the reaction was terminated by the addition of 1 M NaOH (50 μl). The absorbance was measured at 405 nm and estimated by using purified Flag-tagged rat caldecrin as the standard.

Preparation of total RNA and RT-PCR. Total RNA was isolated from the caldecrin plasmid injected femoral muscles. Total RNAs were extracted with GenElute mammalian total RNA mini prep kit (Sigma-Aldrich) according to the manufacturer's protocol. Total RNA was reverse-transcribed using the reverse transcription system (Promega, Madison, WI, USA). PCR amplification was performed with the following primer sets: rat caldecrin, forward (5'-TGCGGCCGCATGTTGGGAATTACGGT-3') and reverse (5'-CCTCGAGTTGTATTTTCTCGTTGATCC-3'); GAPDH, forward (5'-ACCACAGTCCATGCCATCAC-3') and reverse (5'-TCCACC ACCCTGTTGCTGTA-3').

Histological analysis. The tissue specimens were prepared from the muscle where the HVJ-E vector was transfected. The tissue specimens were dissected, quickly frozen in liquid nitrogen and sectioned by cryostat. After brief fixation with methanol, caldecrin

Table I. *Effect of caldecrin on BUN concentration of dystrophic mice.*

	BUN (mg/dl)		
Time after injection	Untreated	Caldecrin-treated	(% of no. treated)
3 h	28.92±2.08	21.02±1.16**	(72.7)
6 h	25.23±1.13	20.47±0. 91**	(81.1)

Eight-week-old dystrophic mice (n=4) fasted overnight were treated with or without administration of caldecrin protein (100 μg/kg body weight) and BUN was measured at the indicated time after injection. $^{**}P<0.01$ *vs*. untreated mice. Values are expressed as the mean±SD.

Table II. *Effect of caldecrin protein administration and gene transfer on BUN concentration of dystrophic mice.*

	Untreated	Cal. protein	Cal. gene
BUN (mg/dl)	22.15±2.70	13.17±2.12*	12.36±5.74**
(%)	(100)	(59.5)	(55.8)

Eight-week-old dystrophic mice (n=3) were treated with or without daily intraperitoneal injection of caldecrin protein (100 μg/kg body weight, Cal. protein) for 4 days or gene (10 μg/head, Cal. gene) injected once in the muscle 5 days before being sacrificed, and fasted overnight before being sacrificed. At the end of the experiment, the serum was collected and used for the BUN assay. $^{*}P<0.05$, $^{**}P<0.01$ *vs*. untreated mice. Values are expressed as the mean±SD.

was reacted with anti-Flag antibody (Sigma-Aldrich) and Alexa Fluor 594-conjugated anti-mouse IgG antibody (Invitrogen Corp., Carlsbad, CA, USA). Fluorescence images were photographed under fluorescence microscopy.

Evans blue dye (EBD, 1% in PBS) was intraperitoneally injected at a dose of 50 μl/10 g of body weight 24 h before sacrifice to evaluate damaged muscle (30). The tissue specimens were obtained from another left femoral muscle which differed from the gene injected-side muscle, fixed in 10% buffered formalin solution, embedded in paraffin and sectioned transversely (5 μm) at the mid portion of the muscle for hematoxylin and eosin (H&E) staining. Alternatively, tissue specimens were frozen at –80˚C and a transverse section (10 μm) was prepared to observe the fluorescence of EBD.

Measurement of blood urea nitrogen (BUN). Serum was collected from mice treated with caldecrin gene or protein at the indicated times as described and BUN was assayed by the Wako BUN-test kit according to the manufacturer's protocol (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Statistical analysis. For statistical analysis, unpaired Student's *t*-test was used. Differences were considered to be statistically significant at $p<0.05$.

Results

Expression and secretion of caldecrin gene in the muscle. An initial experiment was performed to determine whether the caldecrin gene is delivered and expressed in the muscle. GenomOne-Neo including the prCaldecrin3×Flag-IRES-hrGFP expression vector was injected in the femoral muscle of normal mice. As shown in Figure 1A, RT-PCR analysis revealed that caldecrin mRNA from the HVJ-E-mediated vector in the muscle was detected for at least 3 days and continued for 7 days but not 14 days after vector injection. Histochemical analysis showed that the GFP (green) was distributed throughout the myofiber, whereas caldecrin3×Flag (red) was located along with the contour of GFP expressed myofiber (Figure 1B). Caldecrin protein is detected in the membrane and/or secrete vesicles in the cell in which GFP was simultaneously expressed under the IRES promoter. Indeed, by the ELISA analysis, caldecrin-Flag protein in the serum of the caldecrin gene-transfected mouse was increased but not in that of mock-transfected mice (Figure 1C). The concentration of caldecrin in the serum reached its detectable maximum level at 3 days after vector injection, and gradually decreased but remained at 14 days even though its mRNA was at an almost undetectable level. These results suggest that the caldecrin gene transferred by GenomOne-Neo is effective for expressing caldecrin for a long period of time in the muscle and expressed caldecrin was secreted from the muscle to the blood.

Caldecrin prevents the progression of muscle dystrophy in dy/dy mice. To investigate whether caldecrin suppresses BUN, recombinant human caldecrin protein (100 μg/kg body weight) was administered by intraperitoneal injection to dystrophia muscularis (*dy*/*dy*) mice. The serum was collected at 3 and 6 h after injection and BUN was determined. Table I indicates that caldecrin suppressed BUN to 70 to 80% as compared with that of PBS-administered control mice. Furthermore, we examined the long-term effects of caldecrin on the reduction of serum BUN in *dy*/*dy* mice. We compared the serum BUN from the *dy*/*dy* mice treated with or without the recombinant caldecrin, or the caldecrin gene. As shown in Table II, serum BUN levels from mice administered caldecrin and the gene transfer were lower as compared with untreated *dy*/*dy* mice.

Next, we studied caldecrin effects on dystrophic muscle pathology. H&E staining of a cross-section of the femoral muscle of normal control mice revealed that muscle fibers were almost regular and a dark-colored nucleus was found peripheral in the muscular fiber (Figure 2A). H&E staining of caldecrin-treated muscle from normal mice did not show any difference in muscular structure as compared to untreated muscle. On the contrary, in *dy*/*dy* mice, the cross-sections of muscle fibers exhibited round contours, marked variability of fiber size diameter, increase of the interstitial connective tissue

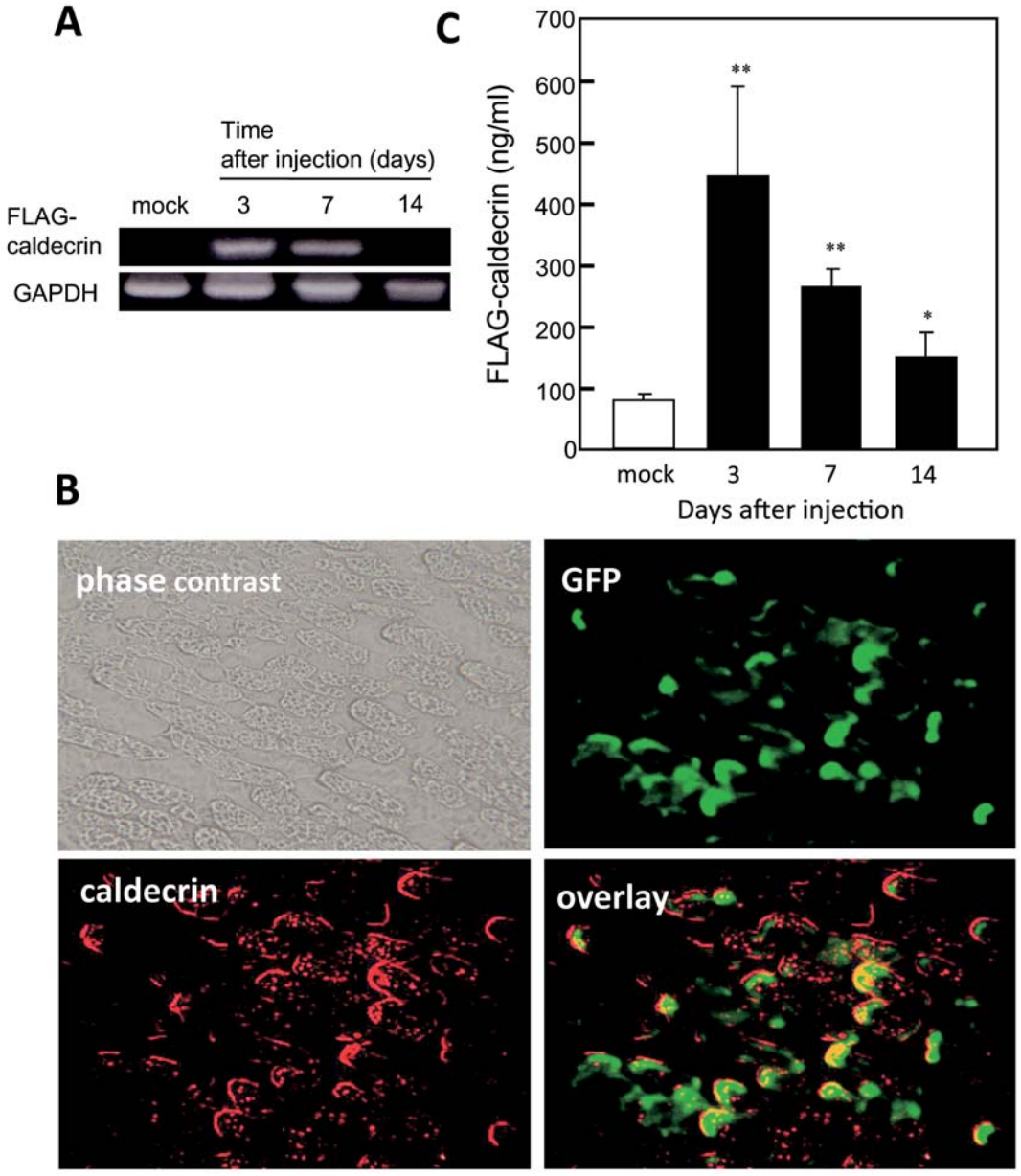

Figure 1. *Ectopic expression of caldecrin in the normal mouse muscle. Time course of caldecrin gene expression in the muscle and protein concentration in the serum after gene transfection in normal mouse muscle. Flag-tagged caldecrin gene in the HVJ envelope was injected into normal mouse muscle. A: At the indicated time, the injected muscle was dissected for caldecrin expression by PCR analysis. B: GFP and flag-caldecrin expressed in the muscle at 7 days after gene injection. Caldecrin gene was transfected into the muscle as described above. A frozen section of the injected femoral muscle was prepared, briefly fixed, and stained with anti-Flag antibody. Fluorescence images of GFP (intracellular gene expression control; green) and caldecrin (red) were photographed using fluorescence microscopy. C: At the indicated time, serum was collected and caldecrin concentration measured by ELISA analysis ($^{*}P<0.05$, $^{**}P<0.01$ vs. mock-treated group).*

and centrally localized nuclei. However, both groups of mice treated with caldecrin protein or its gene revealed that the basic structure of the muscular tissue was well maintained, and the number of centrally located nuclei (19.0% in caldecrin administration, 24.1% in gene expression) was lower as compared with untreated *dy/dy* control (27.8%).

EBD binds albumin, fluoresces red and can enter cells with damaged plasma membrane. EBD is widely used to detect membrane permeability of myofiber and is an *in vivo* marker of myofiber damage (30). To assess the effects of caldecrin on muscular regeneration, EBD exclusion analysis was performed and normal control mice and dystrophic mice

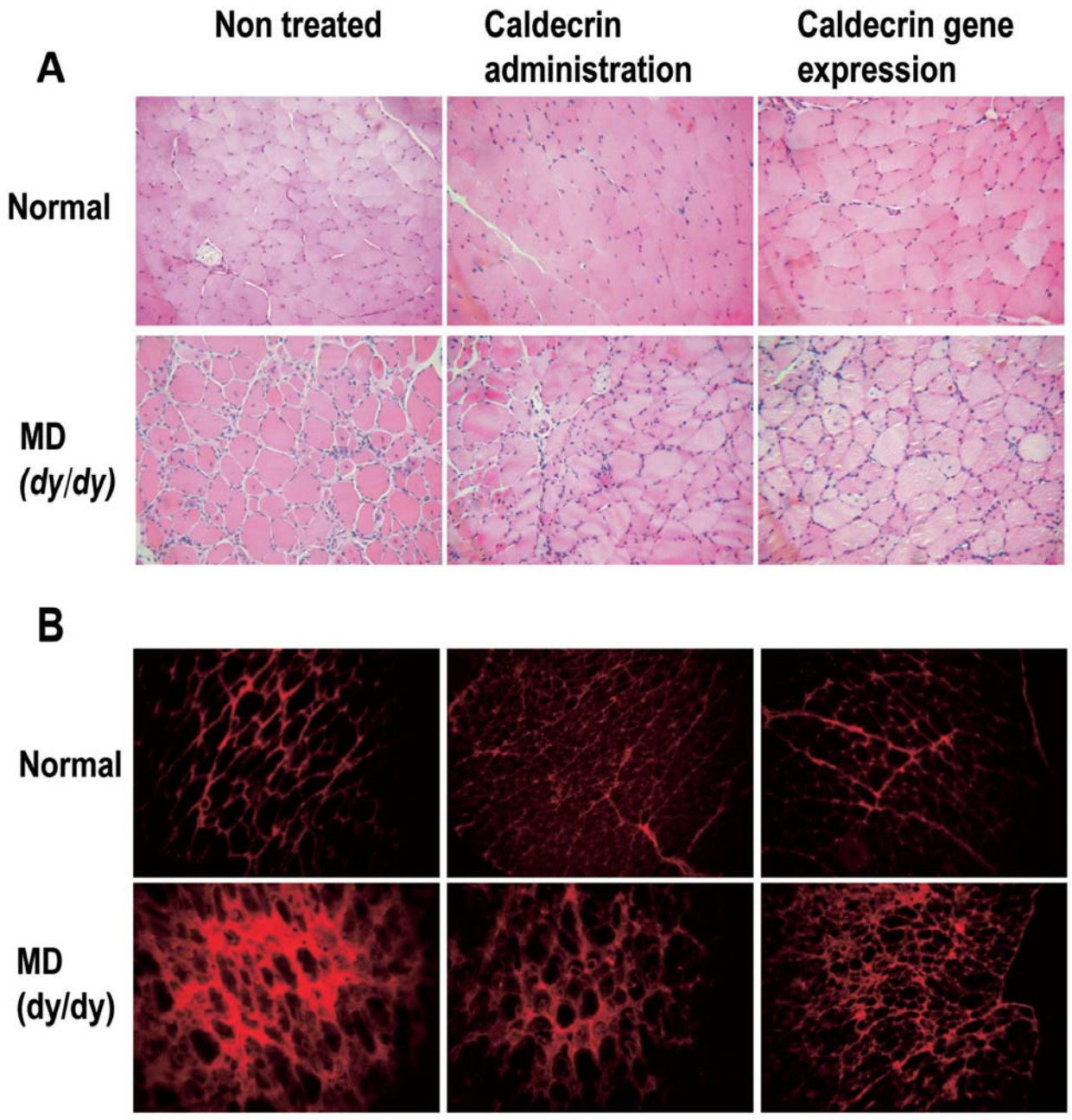

Figure 2. *Effect of caldecrin on muscle from muscular dystrophic and control mice. A: H&E staining of the femoral muscle from muscular dystrophic (dy/dy) and control mice treated with or without recombinant caldecrin or ectopic caldecrin gene. The femoral muscles were dissected after treatment with caldecrin as described in the Materials and Methods. B: Dystrophic and normal mice were treated with or without caldecrin as described in the Materials and Methods. Evans blue dye was injected into the muscular dystrophic and normal mice 10 h before sacrifice. The femoral muscle was dissected and a frozen section was prepared. Fluorescence images of Evans blue dye were taken by fluorescence microscopy.*

were compared (Figure 2B). Autofluorescence caused by EBD was seen around few muscular fibers of normal mice. On the other hand, in muscular tissue of the *dy/dy* mice, EBD penetrated widely into the muscular fiber, indicating that membrane damage had occurred. Interestingly, both caldecrin administration and its gene expression prevented the penetration of EBD. Taken together these results indicated that administration of the caldecrin protein and ectopic gene expression protected *dy/dy* mice from muscular dystrophy.

Discussion

In this study, we demonstrated the therapeutic effect of caldecrin on the muscular damage in muscular dystrophic *dy/dy* mice. The pioneering work of Takaoka *et al*. showed that Px possesses protective components for muscular dystrophy of FSHD and LGMD. This potency is correlated with serum calcium- and BUN-decreasing activities (19-20). Caldecrin also suppressed not only serum calcium concentration (23) but also BUN (in this report). Therefore, we assumed that caldecrin might be one of the active components of Takaoka's Px. BUN is the result of progression under protein catabolism caused by *e.g*. burns, fever, steroid use and muscular dystrophy. The reduced level of BUN with caldecrin treatment may result from protective effects on protein catabolism in the muscle of *dy/dy* mouse. Regarding the serum calcium-decreasing activity, caldecrin inhibits bone resorbing-osteoclast cell formation (27) and activation (26), which may result in lowering the Ca^{2+} concentration in the serum.

At present, the precise molecular mechanism of the protective effects of caldecrin on the muscle dystrophy is unclear. Caldecrin may play a role in the modulation of the Ca^{2+} signaling pathway. Recently, we found caldecrin inhibits RANKL-induced osteoclastogenesis by suppression of NFATc1 activity though PLCγ1-mediated Ca^{2+} oscillation (27). Furthermore, caldecrin suppressed Ca^{2+} influx in mature osteoclast (unpublished data). It is postulated that the mutation of extracellular or intracellular structure proteins in the disease of muscular dystrophy results in cell membrane instability, initiating an influx of Ca^{2+} though the sarcolemma (31-34), which leads to increased proteolysis and muscle injury. Calcium channel blocker diltiazem responds to FSHD (35). Caldecrin may suppress the elevation of intracellular calcium in the muscle fiber of dystrophic mice. The other possibility of the caldecrin signaling pathway on the protective effects on the muscular dystrophy is considered in a manner similar to insulin-like growth factor (IGF). IGF protected against muscular dystrophy to stimulate the regeneration of satellite cells through activation of the PI3K/Akt pathway (36-37). Central nucleation reflects ongoing degeneration and regeneration occurring in dystrophic muscular cells. The number of centrally localized nuclei was lower in the muscle of the caldecrin-treated *dy/dy* mice compared to that of untreated *dy/dy* mice (Figure 2), suggesting that primary effects of caldecrin might be a protection against acute periods of muscular damage rather than the promotion of regeneration of muscle cells. Molecular mechanisms of caldecrin therapeutic effects, including its exertion on calcium homeostasis in the muscle, are expected to be clarified in the future.

In conclusion, caldecrin is secreted into the blood from muscle transfected with caldecrin gene and the progression of muscular dystrophy was reduced. Caldecrin is not only a potential novel molecular tool for understanding the mechanisms of the pathogenesis of muscular dystrophy, but also a therapeutic tool for dystrophic muscle.

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