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Activation of Caspase-3 Apoptotic Pathways in Skeletal Muscle Fibers in Laminin α2-Deficient Mice

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***dy/dy* mice, which carry an unidentified mutation in the Lama2 gene, show dystrophic pathologies similar to those of human congenital muscular dystrophy. Laminin α2 deficiency induces apoptosis with DNA fragmentation. Caspases, which are involved in various types of cell death, are sequentially activated through a processing by other members of caspases. By using a cleavage site-directed antibody against caspase-3 that specifically reacts with the active form of caspase-3, we immunochemically demonstrated that caspase-3 is activated in the skeletal muscle fiber of *dy/dy* mice and that some of the activated caspase-3 muscle fibers are TUNEL-positive. Thus the lack of laminin α2 signals activates caspase-3, resulting in the apoptosis of muscle fibers.** © 1999 Academic Press

Congenital muscular dystrophy (CMD) is divided into two major categories (1): Fukuyama type congenital muscular dystrophy (FCMD) and non-FCMD consisting of two subgroups, laminin α2-negative and -positive CMD. Specific mutations in the gene (*LAMA2*) encoding laminin α2 are found in human laminin α2-negative CMD patients with severely defective muscle basement membranes (2). *dy/dy* mice, carrying an unidentified mutation in the *Lama2* gene, also show dystrophic pathologies (3), and are a murine model for human laminin α2-negative CMD. Recent studies have shown that a lack of laminin α2 results in apoptotic cell death in myogenic cells *in vitro* (4) and *in vivo* (5, 6). Apoptotic cell death is also found in the skeletal muscle fibers of *mdx* (7–9) and γ-dystroglycan-deficient mice (10). Thus, a close relation between apoptotic cell death and dystrophic pathologies has been shown. However, little is known about the molecular mechanism by which apoptotic cell death occurs in the skeletal muscle fiber of mice with dystrophic pathologies.

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The caspase family, a homologue of *C. elegans* Ced-3, is involved in various types of cell death (11). Caspases are activated by sequential processing by family members (12); for instance, caspase-3, which is the farthest downstream in this cascade, is activated by processing of procaspase-3 (p32) into its active form (p20/17 and p12) by upstream caspases during apoptosis (13). Caspase-9, upstream of caspase-3, is autoprocessed into active form by association with cytochrome c/Apaf-1 (14), a homologue of Ced-4, resulting in the activation of caspase-3 (15). Thus, activation of caspase-9 and -3 is a key process in apoptosis.

In contrast with *in vitro* condition, it is difficult to biochemically and immunohistochemically detect the activation of caspases in tissues undergoing apoptosis *in vivo* because apoptotic cells occur infrequently *in vivo* and because the expression of procaspases does not implicate the activation of caspases. Recently, we successfully used cleavage site-directed antiserum against caspase-3 (anti-p20/17), which reacts specifically with active form of caspase-3 (16–18) to detect the activated caspase-3 in naturally occurring cell death. In the present study, we performed immunostaining with anti-p20/17 and terminal-deoxytransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) to demonstrate that the caspase-9 and -3 apoptotic pathways are activated in skeletal muscle fibers of laminin α2-deficient mice.

MATERIALS AND METHODS

Mice. Homozygous (*dy/dy*) mutant mice prepared by *in vitro* fertilization were kindly supplied by Central Institute for Experimental Animals (Kanagawa, Japan).

Preparation of antiserum against the cleavage site of caspases. Antiserum against cleavage site of caspase-3, anti-p20/17, was prepared as described elsewhere (16–18). A peptide corresponding to putative C-terminal processing site of human caspase-9 (19) plus cysteine, CFDQLD, was synthesized (Sawady, Tokyo). Antiserum against activated caspase-9 was generated by injecting CFDQLD conjugated to keyhole limpet hemocyanin (KLH) into rabbits. Specific antibodies against cleavage site of caspase-3 and -9 were purified by CGIETD- and CFDQLD-peptide affinity column chromatog-

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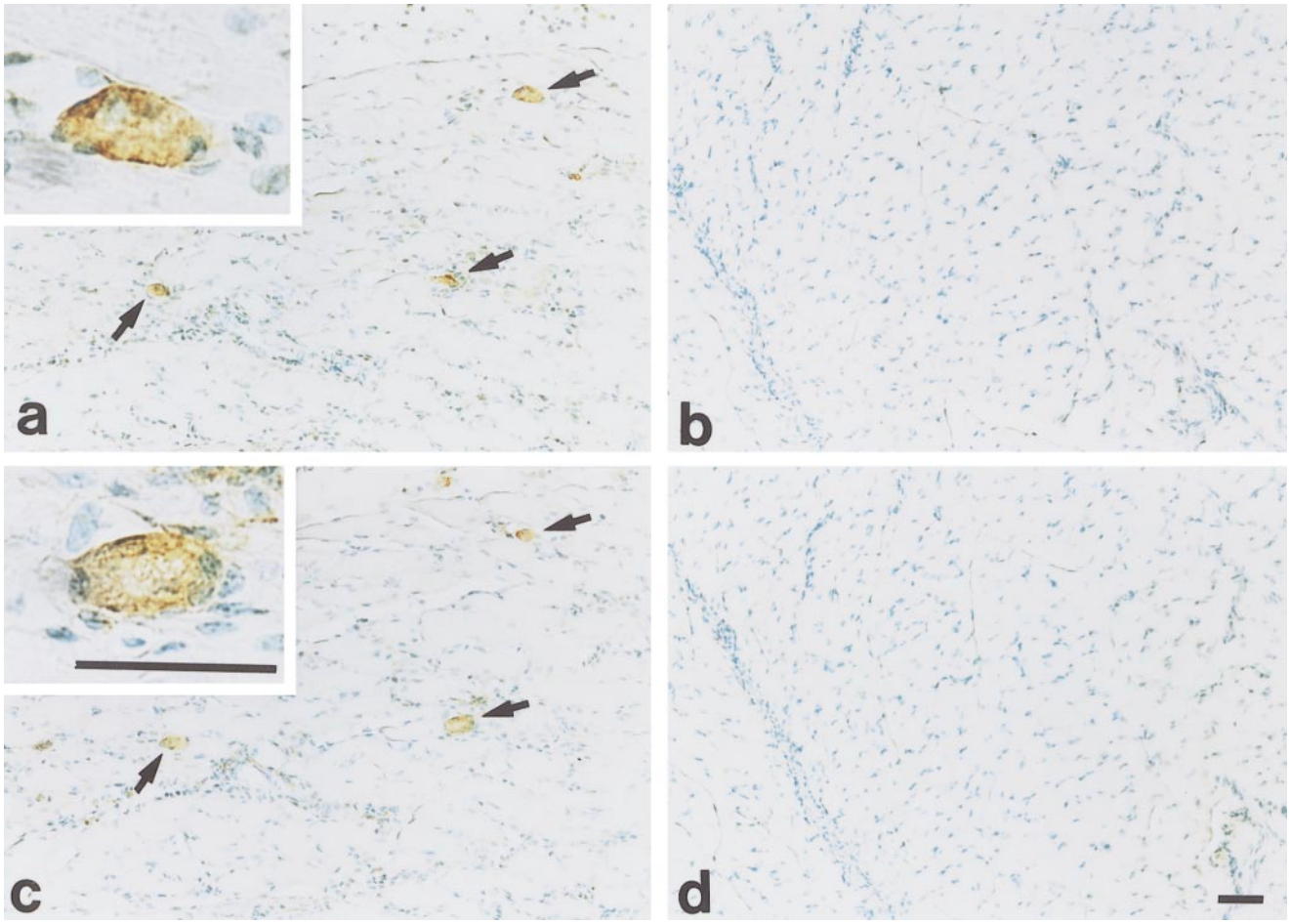

FIG. 1. Detection of activated caspase-9 and activated-3 in skeletal muscle of *dy/dy* mice. Sections of gastrocunemius muscle of 2 week-old *dy/dy* (a, c) and B6 mice (b, d) are immunostained by anti-p20/17 (a, b) or anti-FDQLD (c, d), respectively. Arrows indicate positive muscle fibers. Bar indicates 100 μm. Rectangles are higher magnification of positive muscle fibers.

raphy, respectively. Anti-FDQLD specifically reacted the processing fragment of caspase-9, but not proform of caspase-9 and other caspases (submitted elsewhere).

Immunohistochemistry. Gastrocunemius muscles of 2 weeks-old *dy/dy* mice and B6 mice were frozen in isopentane cooled in liquid nitrogen. Serial frozen sections (6-μm thick) were cut on a cryostat and attached to slides coated with VECTABOND reagent (Vector Laboratories, Burlingame, CA). The sections were subjected to immunostaining using anti-FDQLD and anti-p20/17. The immunoreactivities were detected by peroxidase-conjugated avidin-biotin kit (Vector Laboratories).

Double-labeling by anti-p20/17 and TUNEL. After the reaction of immunostaining was stopped by washing the sections with distilled water, they were subjected to the TUNEL using Apotag kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions. The digoxigenin-(DIG) labeled DNA was detected by alkaline phosphatase-conjugated anti-DIG Fab fragments and 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP).

RESULTS AND DISCUSSION

Recently, the apoptosis of epithelial and endothelial cells induced by detachment from basal membrane has been categorized as anoikis (20). Caspase-1 (ICE) is activated by detachment from basal membrane (21). Integrins are involved in the survival signals from laminin, a component of the basal membrane (22). Integrin $\alpha 7\beta 1$, a receptor for laminin $\alpha 2$, also plays a central role in transducing basal membrane-skeletal muscle cell signals (23). Antibody against integrin $\beta 1$D induces the apoptosis of myotube, which is inhibited by overexpression of Bcl-2, a homologue of Ced-9 and upstream of caspases (5), suggesting that caspases are involved in apoptosis of muscle fibers induced by laminin $\alpha 2$-deficiency.

Immunostaining using anti-p20/17 revealed that p20/17-positive muscle fibers were present in the *dy/dy* mice but were undetectable in the B6 mice (Figure 1a and b). More than 1% of skeletal muscle fibers were positive in *dy/dy* mice but less than 0.001% were positive in B6 mice. Moreover, immunostaining of serial sections by anti-p20/17 and anti-FDQLD, antisera against the cleavage site of caspase-9, revealed that all

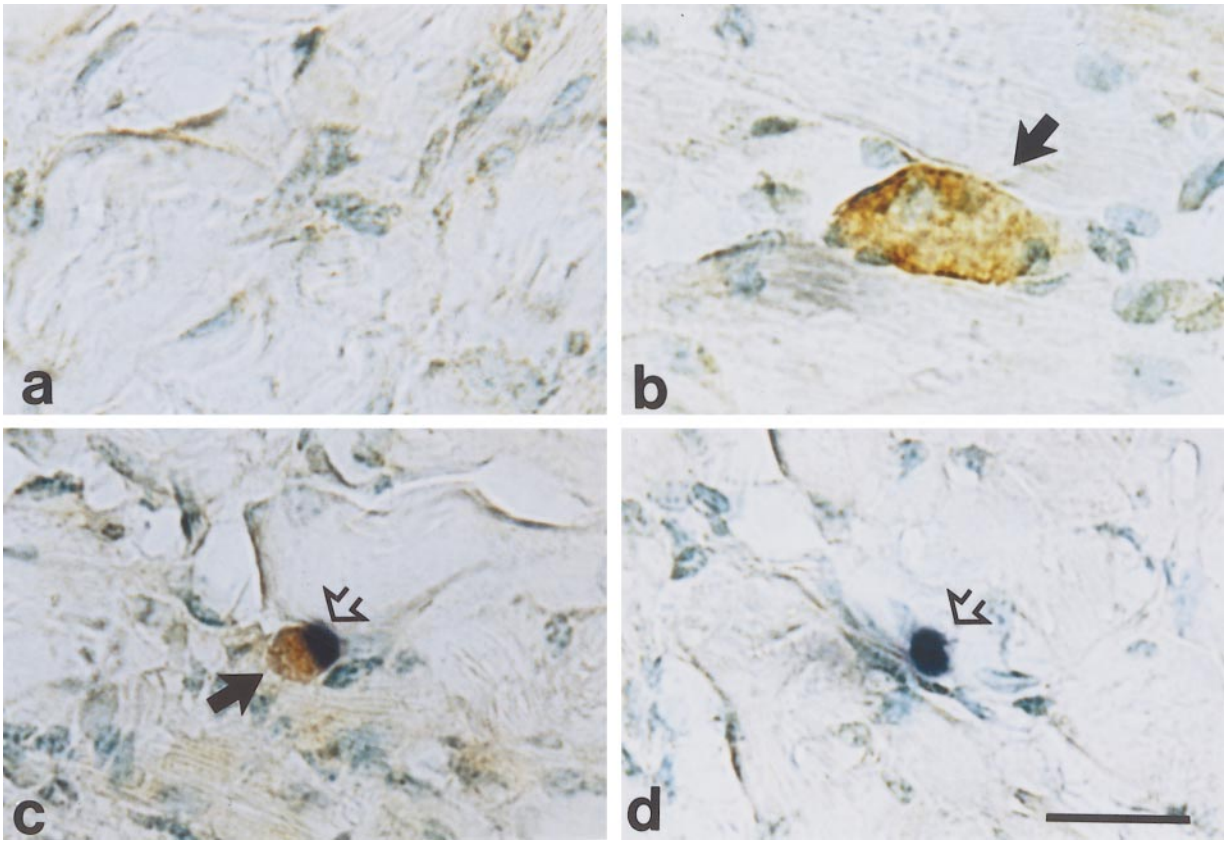

FIG. 2. Double-staining of muscle fibers of *dy/dy* mice by TUNEL and immuno-staining using anti-p20/17. (a) p20/17-negative and TUNEL-negative muscle fibers, (b) a p20/17-positive and TUNEL-negative muscle fiber, (c) a p20/17-positive and TUNEL-positive muscle fiber, (d) a p20/17-negative and TUNEL-positive muscle fiber. Closed arrows indicate p20/17-positive muscle fibers and open arrows indicate TUNEL-positive muscle fibers. Bar indicates 100 μm.

p20/17-positive muscle fibers were positive for anti-FDQLD (Figure 1a and c). Thus, caspase-3 and caspase-9 were processed into active form in the same muscle fibers of *dy/dy* mice. At present, it is not yet clear whether absence of laminin α2 signals activates caspase-3 via activation of caspase-9 or independently activates caspase-3 and caspase-9 in the same muscle fibers. However, caspase-3 is not activated in caspase-9-deficient mice (24), which suggests that the former possibility is more likely.

Activated caspase-3 muscle fibers and apoptotic muscle fibers in *dy/dy* mice were double-stained by immunostaining using anti-p20/17 and TUNEL staining (Figure 2). Some of the p20/17-positive muscle fibers were TUNEL-positive (Figure 2c). Thus, the absence of laminin α2 signals is closely related to the activation of caspase-9 and caspase-3, which results in DNA fragmentation *in vivo*. However, p20/17-positive and TUNEL-negative muscle fibers or p20/17-negative and TUNEL-positive muscle fibers were detected in *dy/dy* mice at frequencies equivalent to those of p20/17-positive and TUNEL-positive muscle fibers (Figure 2b and d). p20/17-positive and TUNEL-negative muscle fibers may be in the process of apoptosis; otherwise, the caspase-3-dependent apoptotic pathway may be blocked in muscle fibers by protective factors such as X-linked inhibitor of apoptosis protein (XIAP) (25). The TUNEL-positive muscle fibers were thin and shrunken (Figure 2c and d). p20/17-negative and TUNEL-positive muscle fibers may be already in the final stage of apoptosis after caspase-3 activation decreased because caspase-3 is transiently activated at the initial stage of apoptosis and then the active form of caspase-3 (p20 or p17) rapidly degrades (26). However, we do not completely exclude the possibility that other caspase-dependent or caspase-independent apoptotic pathways are also activated in *dy/dy* mice.

Bcl-xL, an antiapoptotic member of Bcl-2, is involved in the negative regulation of activation of caspase-3 as a suppressor factor interacting with caspase-9 and Apaf-1 (27, 28). The activation of caspase-9 is also regulated by the balance of phosphorylation and dephosphorylation of Bad, an apoptotic member of Bcl-2 family, by survival factors through phosphatidylinositol-3 kinase (PI3K)/Akt signals (29). Downstream of integrin signals involved in cell survival is PI3K/Akt (30). Upon engagement of integrins in the interaction with basement membranes, PI3K/Akt is constitutively

activated to promote cell survival. Thus, absence of laminin/integrin signals might induce dephosphorylation of Bad to decrease the Bcl-xL homodimers, which results in the activation of caspase-9 and caspase-3. Otherwise, it might induce mitochondrial membrane damage to release cytochrome c, which activates caspase-9 through association with Apaf-1 (14). Growth factors and oncogene products, which can substitute for the integrin signals by activating PI3K/Akt signals (31), or protective factors such as Bcl-xL might prevent apoptosis of the muscle fibers in laminin α2-deficient mice. This could explain the nonsimultaneous activation of caspase-9 and caspase-3 in muscle fibers of the laminin α2-deficient mice. The small percentage of apoptotic muscle cells with non-simultaneous activation of caspases could explain non-progressive nature of laminin α2-negative muscular dystrophy.

We do not exclude the involvement of dystroglycan, another receptor for laminin α2, in the apoptosis of muscle fibers of subjects with dystrophic pathologies (10). However, we could not detect the activation of caspase-3 or caspase-9 in the apoptotic muscle fibers of *mdx* mice by immunostaining. Laminin-dystroglycan-dystrophin signals may be involved in the survival signals of skeletal muscle; however, their protection might be against other apoptotic pathways than that of caspase-3 or caspase-9. We are currently investigating the involvement of activation of other caspases in apoptosis in *dy/dy* and *mdx* mice.

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