Inhibition of apoptosis improves outcome in a model of congenital muscular dystrophy

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The most common form of human congenital muscular dystrophy (CMD) is caused by mutations in the laminin-α2 gene. Loss of laminin-α2 function in this autosomal recessive type 1A form of CMD results in neuromuscular dysfunction and, often, early death. Laminin-α2–deficient skeletal muscles in both humans and mice show signs of muscle cell death by apoptosis. To examine the significance of apoptosis in CMD1A pathogenesis, we determined whether pathogenesis in laminin-α2–deficient (_Lama2_$^{-/-}$) mice could be ameliorated by inhibiting apoptosis through either (a) inactivation of the proapoptosis protein Bax or (b) overexpression of the antiapoptosis protein Bcl-2 from a muscle-specific transgene. We found that both of these genetic interventions produced a several-fold increase in the lifespan of _Lama2_$^{-/-}$ mice. Bax inactivation also improved postnatal growth rate and myofiber histology and decreased fixed contractures of _Lama2_$^{-/-}$ mice. Thus, Bcl-2 family–mediated apoptosis contributes significantly to pathogenesis in the mouse model of CMD1A, and antiapoptosis therapy may be a possible route to amelioration of neuromuscular dysfunction due to laminin-α2 deficiency in humans.

Introduction

The most common form of human congenital muscular dystrophy (CMD) is caused by mutation of the human _LAMA2_ (mouse _Lama2_) gene that encodes laminin-α2 (1–3). Loss of laminin-α2 function in this autosomal recessive type 1A form of CMD results in neuromuscular dysfunction and often early death. Multiple mutations have been found that lead to truncated gene products and loss of laminin-α2 protein (2, 3), with most of the mutations in exons 1–31 of the 64 exons in the gene. Additional _LAMA2_ mutations, clustered in exons 58–64, produce partial laminin-α2 deficiency with a less severe clinical course (2). Partial laminin-α2 deficiency is also found in Fukuyama CMD, muscle-eye-brain disease, and Walker-Warburg syndrome, though these diseases are caused by mutations in genes other than _LAMA2_ (3).

Laminin-α2 is an extracellular protein with multiple-binding partners. In skeletal muscle, laminin-α2 associates with the additional laminin subunits β1 and γ1 to form the heterotrimeric laminin-2 complex. Heterotrimeric laminins that include laminin-α2 (e.g., α2, β1, γ1), are termed merosins. Thus, CMD1A has also been termed merosin-deficient CMD. Laminin-α2 plays a key role in linking cellular and extracellular components. An extracellular binding partner of laminin-α2 is entactin/nidogen, which in turn binds to collagen IV. In addition, laminin-α2 has at least two membrane-associated binding partners in skeletal muscle: α-dystroglycan and α7-integrin (2), each of which is complexed with additional proteins.

Inappropriate induction of apoptotic cell death in skeletal muscles and motor nerves is one possible mechanism by which loss of laminin-α2 leads to neuromuscular dysfunction. Indeed, laminin-α2–deficient skeletal muscles in both humans and mice show relatively abundant signs of muscle cell death by apoptosis (4–6). To examine the significance of apoptosis in CMD1A pathogenesis, we determined whether pathogenesis in laminin-α2–deficient (_Lama2_$^{-/-}$) mice could be ameliorated by inhibiting apoptosis through either (a) inactivation of the proapoptosis protein Bax or (b) overexpression of the antiapoptosis protein Bcl-2 from a muscle-specific transgene. We found that both of these genetic interventions produced a several-fold increase in the lifespan of _Lama2_$^{-/-}$ mice. Bax inactivation also improved postnatal growth rate and myofiber histology and decreased fixed contractures of _Lama2_$^{-/-}$ mice. We conclude that Bcl-2 family–mediated apoptosis contributes significantly to pathogenesis in the mouse model of CMD1A. In addition, antiapoptosis therapy may be a possible route to amelioration of neuromuscular dysfunction due to laminin-α2 deficiency in humans.

Results

A large percentage of _Lama2_$^{-/-}$ mice die within the first 2 months of birth (5, 7). We found, however, that this early death was completely eliminated when the _Lama2_$^{-/-}$ mice were made _Bax_-null by cross-breeding (Figure 1A). When not sacrificed earlier for histology, we found that all _Lama2_$^{-/-}Bax^{-/-}$ mice that lacked both laminin-α2 and Bax survived for more than 120 days after birth (_n_ = 10), whereas more than half of the _Lama2_$^{-/-}Bax^{+/+}$ mice that lacked laminin-α2 but had normal levels of Bax died within 60 days of birth. We also found an improvement in lifespan of _Lama2_$^{-/-}$ mice that were _Bax_$^{+/-}$ heterozygotes and thus had reduced expression of Bax, though this improvement was modest compared with the complete elimination of early death seen in _Lama2_$^{-/-}$ mice that entirely lacked Bax (Figure 1A). We kept _Lama2_$^{-/-}Bax^{-/-}$ mice for up to 171 days prior to sacrifice for histological studies and never saw a spontaneous death during this period.

In addition to increasing lifespan, inactivation of Bax expression improved the growth of _Lama2_$^{-/-}$ mice (Figure 1, B and C). _Lama2_$^{-/-}Bax^{-/-}$ mice gained weight more quickly and grew significantly larger than _Lama2_$^{-/-}Bax^{+/+}$ mice; nonetheless, the _Lama2_$^{-/-}Bax^{-/-}$ mice did not grow as quickly or to as large a size as healthy mice that had normal expression of laminin-α2. Although absence of Bax improved the growth of _Lama2_$^{-/-}$ mice, the presence or absence of Bax did not affect the growth rate of normal _Lama2_$^{+/+}$ mice (8).
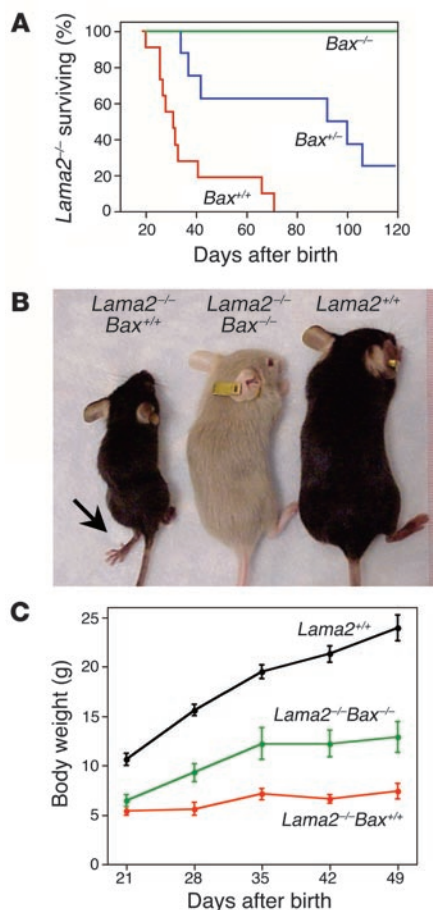
Figure 1

Inactivation of Bax eliminated early death and improved growth of laminin-α2–deficient *Lama2^{−/−}* mice. (**A**) Survival curves for *Lama2^{−/−}* mice show that most *Lama2^{−/−}Bax^{+/+}* mice died within 2 months of birth, whereas lifespan was modestly increased in heterozygous *Lama2^{−/−}Bax^{+/−}* mice, and early death was completely eliminated in *Lama2^{−/−}Bax^{−/−}* mice. (**B**) Pictured at 6 weeks after birth, a *Lama2^{−/−}Bax^{−/−}* mouse (center) was much larger than a *Lama2^{−/−}Bax^{+/+}* mouse (left), though not so large as a wild-type *Lama2^{+/+}Bax^{+/+}* mouse (right). Contractures of hind limbs (arrow) developed in *Lama2^{−/−}Bax^{−/−}* mice during the first 2–3 months of life but did not develop on *Lama2^{−/−}Bax^{−/−}* or wild-type mice. (**C**) Growth curves show that the postweaning growth rate of *Lama2^{−/−}* mice was significantly increased by inactivation of Bax, through growth of *Lama2^{−/−}Bax^{−/−}* mice lagged behind that of healthy *Lama2^{+/+}* wild-type mice. Error bars = SE; n = 5–15. The mean body weights of *Lama2^{−/−}Bax^{−/−}* and *Lama2^{−/−}Bax^{+/+}* mice were significantly different from each other with $P < 0.04$ at days 28 and 35 and $P < 0.01$ at days 42 and 49.

The lifespan of *Lama2^{−/−}* mice was also increased several-fold by transgenic overexpression of the antiapoptosis protein Bcl-2 in skeletal muscle. To increase expression of Bcl-2 only in skeletal muscle, we produced a transgene in which an approximately 7-kb fragment of the muscle-specific MyoD promoter was used to direct expression of human Bcl-2 (Figure 2A). Bcl-2 is not highly

expressed in normal adult skeletal muscle (9) (Figure 2B). Expression of human Bcl-2 by the *pMyoD-hBcl-2* transgene was restricted to skeletal muscle and not observed in other tissues (Figure 2B). When introduced into *Lama2^{−/−}* mice by crossbreeding, this *pMyoD-hBcl-2* transgene increased lifespan, because all the transgenic mice (n = 6) lived longer than 90 days. Of these 6 transgenic *Lama2^{−/−}* mice, we noted deaths at 94, 132, and 147 days, with 3 remaining alive at 120 days as of this writing. The lifespan of transgenic mice was thus much longer than that of nontransgenic *Lama2^{−/−}* mice. The *pMyoD-hBcl-2* transgene also increased the growth rate of *Lama2^{−/−}* mice. At 4–4.5 weeks of age, male *Lama2^{−/−}* mice with the *pMyoD-hBcl-2* transgene weighed 10.2 ± 2.5 g (average ± SD, n = 9), whereas those without the transgene weighed significantly less at 6.3 ± 2.3 g (n = 7, $P < 0.01$ by *t* test). The ability of the transgene to increase survival and growth of *Lama2^{−/−}* mice shows that skeletal muscle-specific alteration of Bcl-2 is sufficient to ameliorate pathology due to laminin-α2 deficiency.

Bax inactivation, but not muscle-specific expression of Bcl-2, also decreased fixed contractures of the hind limbs in *Lama2^{−/−}* mice. In addition to skeletal muscle defects, most *Lama2^{−/−}* mice develop fixed contractures (i.e., permanent locking into an extended position) of the hind limbs by 6–8 weeks after birth (Figure 1B), apparently due

Figure 2

(**A**) Diagram of the cDNA used to produce *pMyoD-hBcl-2* transgenic mice in which an approximately 7-kb fragment of the mouse MyoD promoter was used to drive expression of full-length human Bcl-2 (hBcl-2) cDNA. Arrows show locations of genotyping PCR primers. SV40, simian virus 40 splicing sequence. (**B**) Transgene expression is skeletal-muscle specific. As indicated, Ab's specific for transgenic human Bcl-2 or endogenous mouse Bcl-2 were used to probe Western blots of protein extracts made from tissues of wild-type or transgenic mice. Human Bcl-2 was expressed only in skeletal muscles (LM, leg muscles; Di, diaphragm) of transgenic mice; it was not expressed in skeletal muscles of wild-type mice or in nonmuscle tissues (Kd, kidney; Br, brain; Ht, heart) of transgenic mice. The endogenous mouse Bcl-2 was expressed in thymus (Th) and spleen (Sp) of wild-type mice, but was not detectable in skeletal muscles of either wild-type or transgenic mice or in heart and liver (Lv) of transgenic mice. (**C**) Myotubes formed in culture from myoblasts derived from 3-week-old *pMyoD-hBcl-2* leg muscles were more resistant to cell death induced by 100 nM staurosporine measured by propidium iodide (PI) permeability than those formed by wild-type myoblasts.
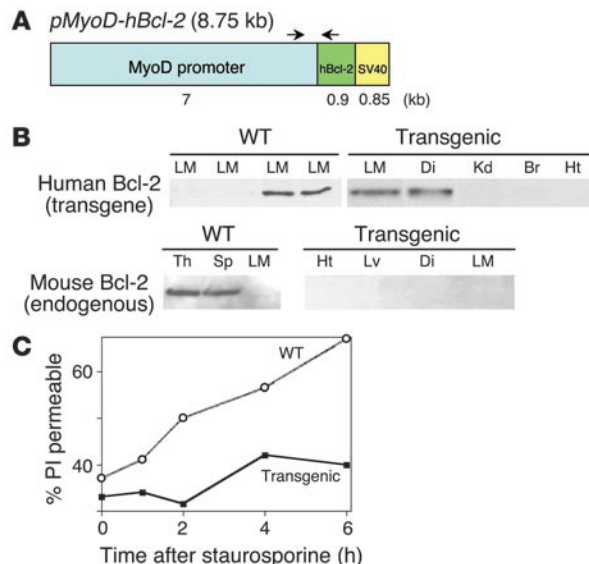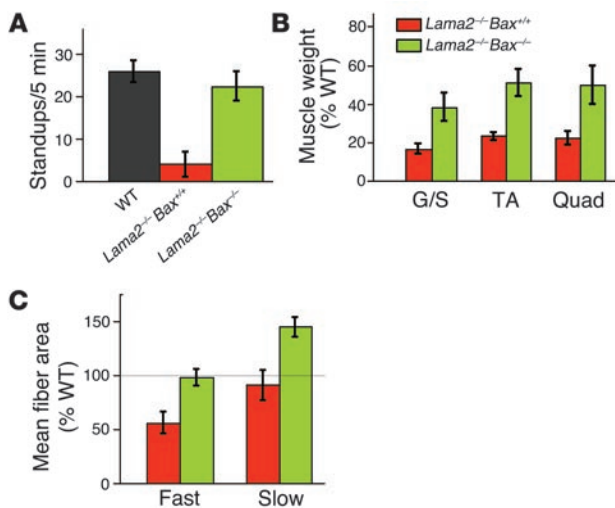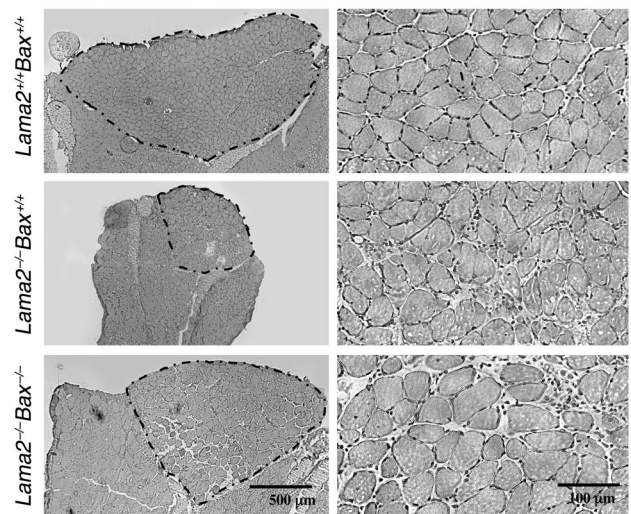
Figure 3

Inactivation of Bax improved *Lama2−/−* muscles. (**A**) Exploratory behavior, measured by the number of times mice stood up on their hind legs during the first 5 minutes in a new cage, was much decreased in 6-week-old *Lama2−/−Bax+/+* mice (red bar), compared with wild-type *Lama2+/+Bax+/+* mice (WT, black bar). When Bax was inactivated in *Lama2−/−* mice, in contrast, the *Lama2−/−Bax−/−* mice (green bar) showed exploratory behavior that was near that of wild-type. Error bars = SE; *n* = 3 for *Lama2−/−Bax+/+* mice and *n* = 4 for WT and *Lama2−/−Bax−/−* mice. (**B**) The mass of individual *Lama2−/−* muscles was increased by inactivation of Bax. The gastrocnemius/soleus (G/S), tibialis anterior (TA), and quadriceps (Quad) muscles weighed more in *Lama2−/−Bax−/−* mice (green bars) than in *Lama2−/−Bax+/+* mice (red bars). Data are plotted as percentage of wild-type (*Lama2*-positive, *Bax*-positive) values. Error bars = SE; *n* = 3–11. (**C**) The size of myofibers in *Lama2−/−* muscles was increased by inactivation of Bax. In the soleus muscle, both fast and slow myofibers had larger mean cross-sectional areas in *Lama2−/−Bax−/−* mice (green bars) than in *Lama2−/−Bax+/+* mice (red bars). Data are plotted as percentage of wild-type (*Lama2*-positive, *Bax*-positive) values. Error bars = SE; *n* = 3–5. In all panels, the *Bax*-positive and *Bax*-null samples were significantly different at *P* < 0.05 (*t* test using primary data prior to conversion to percentage of wild-type).

to failure of basal lamina formation by Schwann cells and subsequent conduction defects in motor nerves (2, 5, 7, 10). Fixed contractures and paralysis are not prevented by muscle-specific expression of laminin-α2 (7). We also observed development of hind limb contractures in *Lama2−/−* mice that had normal Bax expression, even if they also carried the muscle-specific Bcl-2 transgene. In marked contrast, we did not observe fixed contractures of the hind limbs in *Lama2−/−* mice that lacked Bax (Figure 1B). Even up to 6 months of age, the hind limbs in *Bax*-null mice, though appearing much weaker than wild-type, were not paralyzed. Thus, inactivation of Bax expression, which is normally widespread in both muscle and nonmuscle tissues (11), appeared to ameliorate functional defect motor nerves, as well as skeletal muscles, of *Lama2−/−* mice.

Inactivation of Bax also increased the spontaneous standing movements of *Lama2−/−* mice, which is further evidence of improved hind limb function. When placed in a new cage, normal mice showed exploratory behavior, including repeated standing up on hind limbs. As early as 4–5 weeks after birth, we found that *Lama2−/−* mice stood up less than 25% as often as normal mice, consistent with impaired ability of the hind limbs to function in weight-bearing activity. This spontaneous standing behavior was

increased to near the level found in normal mice, however, when the *Lama2−/−* mice were made *Bax*-null (Figure 3A).

Individual muscles in *Lama2−/−* mice that were also *Bax* null were bigger and had larger and more myofibers than did muscles in *Lama2−/−* mice that were *Bax* positive (Figure 3, B and C, and Figure 4). Compared with the healthy muscles in *Lama2+/+* mice, the diseased muscles in *Lama2−/−* mice were much smaller. When the *Lama2−/−* mice were made *Bax*-null, however, the weights of individual muscles (e.g., gastrocnemius/soleus complex, tibialis anterior, quadriceps) were increased (Figure 3B). The cross-sectional areas of the muscles also increased; for example, in the soleus muscle (Figure 4) the mean cross-sectional area (± SE) of the muscle was 0.47 ± 0.04 mm^2 ($n = 3$) in *Lama2−/−Bax+/+* mice but was increased to 1.14 ± 0.15 mm^2 ($n = 3$) in *Lama2−/−Bax−/−* mice (a significant difference at $P = 0.013$ by the t test). In wild-type mice, the cross-sectional area of the soleus was 1.71 ± 0.16 mm^2 ($n = 6$). The mean cross-sectional areas of both fast and slow myofibers were also significantly increased when the *Lama2−/−* mice were made *Bax*-null (Figure 3C). In addition, the number of myofibers in a muscle was increased when the *Lama2−/−* mice were made *Bax*-null. In the soleus muscle, for example, the mean number (± SE) of myofibers was 336 ± 29 ($n = 3$) in *Lama2−/−Bax+/+* mice, but was increased to 462 ± 3.1 ($n = 3$) in *Lama2−/−Bax−/−* mice, a significant difference at $P = 0.012$ by the t test. In wild-type mice, the mean number of myofibers in the soleus was 991 ± 40 ($n = 6$). Thus, the improvements in *Lama2−/−* muscles produced by inactivation of Bax, though significant, were partial as muscle properties generally remained below normal.

Figure 4

Inactivation of Bax improved soleus muscle histology. Frozen sections were prepared from the leg muscles of 7-week-old mice of different genotypes, including wild-type mice (*Lama2+/+Bax+/+*, top row), laminin-α2–deficient mice with normal Bax (*Lama2−/−Bax+/+*, central row), and laminin-α2–deficient mice with no Bax (*Lama2−/−Bax−/−*, bottom row) as indicated. After staining with H&E, sections were examined at low (left column) and high (right column) magnification. In the left column, the soleus muscles are outlined to show that inactivation of Bax increased the overall size of the *Lama2−/−* soleus muscle, and the right column shows that inactivation of Bax also increased the cross-sectional areas of myofibers in *Lama2−/−* soleus muscle (see text and Figure 3 for quantitative measurements). Irrespective of *Bax* genotype, *Lama2−/−* soleus muscles had about the same number of mononucleate cells and myofibers with central nuclei. Scale bars: left column, 500 μm; right column, 100 μm.

These improvements in muscle properties upon Bax inactivation were not due to an effect on myofiber formation during development, because when normal $Lama2^{+/+}$ mice are made Bax-null, there is no change in the number of myofibers per muscle and myofibers are somewhat smaller than in Bax-positive, $Lama2$-positive mice (8). The improved muscle properties also did not appear to be due to correction of the altered membrane protein composition found in $Lama2^{-/-}$ mice. For example, we found by Western blotting that the elevation (increased approximately 3 times) of β-dystroglycan seen in $Lama2^{-/-}$, compared with wild-type muscles (12), was not corrected by Bax inactivation (not shown).

Regeneration in $Lama2^{-/-}$ muscles, as measured by myofibers with central nuclei, also did not appear to be altered by inactivation of Bax. Despite significant myopathy, $Lama2^{-/-}$ muscles at 5–8 weeks of age show relatively poor regeneration (13, 14). Newly regenerated myofibers, which have their nuclei located in the center of the fiber rather than at the periphery, amounted to approximately 20% of the total $Lama2^{-/-}$ myofibers in the soleus and other muscles (13, 14). (For comparison, muscles in dystrophin-deficient mdx mice show high levels of regeneration with greater than 90% of myofibers reported to have central nuclei by 24 weeks of age; see ref. 13.) Inactivation of Bax failed to produce any consistent difference in the percentages of myofibers with central nuclei in $Lama2^{-/-}$ muscles (not shown).

Satellite cell numbers also appeared to be unaffected by inactivation of Bax or expression of the transgene. We examined satellite cells in $Lama2^{-/-}$ muscles because both Bax and the MyoD transgene were expressed in proliferating satellite cells, as well as in skeletal muscle cells beginning near the time myofibers first form in embryonic development, and in all multinucleate myofibers in the adult (15, 16) (Figure 2B and data not shown). Nonetheless, neither inactivation of Bax nor expression of the Bcl-2 transgene appeared to affect the number of satellite cells in $Lama2^{-/-}$ muscles, as determined either by counting mononucleate cells found in cross-sections of $Lama2^{-/-}$ muscles or by determining the number of satellite cells that could be successfully cultured per gram of muscle wet weight (data not shown). On the other hand, both Bax inactivation and expression of the Bcl-2 transgene inhibit experimentally induced cell death of both myoblasts and myotubes in culture (8) (Figure 2C, and data not shown).

Discussion

Because $Lama2^{-/-}$ pathology was ameliorated by 2 genetic interventions, inactivation of Bax and overexpression of Bcl-2, that were designed to inhibit apoptosis, it appears that apoptosis plays a significant role in disease progression in $Lama2^{-/-}$ mice. Taken together, our analyses suggest that Bax inactivation does not lessen myopathy in $Lama2^{-/-}$ mice by improving the success of regeneration, but rather by increasing the postnatal survival of myofibers that had formed during earlier stages of muscle development. In skeletal muscle, the heterotrimeric laminins 2 and 4, which include laminin-α2 as a subunit, interact both with basal lamina components and with the cell surface proteins α-dystroglycan and integrin α7/β1 (2). One consequence of the loss of laminin-α2, therefore, is myofiber dysfunction due to disrupted cross-linking of the plasma membrane and basal lamina (17). A second consequence of the loss of laminin-α2, however, could be disrupted integrin and dystroglycan signaling, which might lead to apoptosis.

Previous studies showed that muscle, but not motor nerve, pathology in $Lama2^{-/-}$ mice can be prevented by muscle-specific

expression of either laminin-α2 or a modified agrin protein that can replace laminin (7, 12). In those studies, restoration of laminin function likely both restored muscle function and eliminated apoptosis in muscle cells. Partial amelioration of 1 or more aspects of laminin-α2 deficiency in mice can also be produced by feeding a 50% protein diet, inactivating the complement system, or by administering IGF-1, clenbuterol, or prednisolone (18–21). Whether these treatments reduce pathology through inhibition of apoptosis remains to be determined.

Our study demonstrates that disease due to loss of laminin-α2 can be significantly ameliorated by interventions designed to inhibit apoptosis. Of particular significance is that inactivation of Bax, which is normally widely expressed, appears to ameliorate both muscle and motor nerve pathology, though neither is completely corrected. An important remaining question is whether antiapoptotic therapies will remain effective if started later in life after onset of disease. Genetic and pharmacological treatments designed to inhibit apoptosis also produce a moderate (10–20%) increase of lifespan in a mouse model of familial amyotrophic lateral sclerosis (22–24). Furthermore, treatment with cyclosporin A inhibits mitochondria-mediated apoptosis and improves muscle histology in collagen VI-deficient mice (25). On the other hand, overexpression of an apoptosis-inhibiting protein in dystrophin-deficient mdx mouse muscles does not appear to ameliorate pathology (26). Thus, apoptosis is more important to pathogenesis in some neuromuscular diseases than in others, and apoptosis appears to play a particularly large role in laminin-α2 deficiency.

In $Lama2^{-/-}$ mice, it is possible that more stringent inhibition of apoptosis, perhaps by higher expression of Bcl-2, or additional antiapoptotic factors, or by inactivation of additional proapoptosis Bcl-2 family members, might further reduce pathology beyond what was seen here. Antiapoptosis therapies based on genetic interventions using agents such as RNA-mediated interface (RNAi) or viral vectors might prove applicable to humans. Pharmacological inhibitors of apoptosis such as caspase inhibitors should also be considered as candidates to ameliorate $Lama2^{-/-}$ disease. An antiapoptosis therapy that significantly slows disease progression in human CMD1A could be of considerable benefit.

Methods

Mice. Heterozygous B6.129 $Lama2^{dy\text{-}W/+}$ mice, which carried the targeted dy-W mutation in the $Lama2$ gene (7), were a gift of Eva Engvall (Burnham Institute, La Jolla, California, USA). Heterozygous C57BL/6-Bax^{tm1Sjk} mice with a targeted mutation in the Bax gene (27) were obtained from The Jackson Laboratory. For ectopic expression of Bcl-2 in muscle cells, we prepared transgenic mice in which an approximately 7-kb fragment of the mouse MyoD promoter (28) directed expression of the full-length human Bcl-2 cDNA (from American Type Culture Collection) (Figure 2A). The transgene is named *pMyoD-hBcl-2* and was maintained in C57BL/6J mice. Littermates were used as controls in all experiments.

Western blotting and immunohistochemistry with the human Bcl-2–specific mAbs 6C8 (BD Biosciences — Pharmingen) or Ab-1 (Oncogene Research Products) was used to confirm that the transgenes were expressed only in skeletal muscle and that the transgenes were expressed in all myofibers (Figure 2B and J.A. Dominov, unpublished observation). Endogenous mouse Bcl-2 was detected with the mouse Bcl-2–specific mAb 3F11 (BD Biosciences — Pharmingen).

To produce $Lama2^{-/-}$ mice with the appropriate Bax or transgenic genotypes for these studies, $Lama2^{+/-}$ heterozygotes were first crossbred with either $Bax^{+/-}$ heterozygotes or with mice carrying the *pMyoD-hBcl-2*

transgene. Double heterozygotes among the progeny of this first breeding were then crossbred, and the progeny were genotyped at weaning (approximately 3 weeks of age) using PCR-based assays of tail DNA to identify *Lama2*$^{-/-}$ progeny that carried the desired *Bax* or transgenic genotypes, as well as littermates to serve as controls. Observations of the effects of *Bax* and transgene genotypes on survival of *Lama2*$^{-/-}$ mice began at weaning when genotypes were determined (Figure 1A).

Mice were observed daily, and weights were measured once or twice per week. Mice were sacrificed if pathology increased to a point where movement was severely impaired and death appeared imminent. Functional capabilities of mice were assayed by determining the number of times that mice stood up on their hind limbs during their first five minutes of exploratory motion after being placed in a new cage. Protocols for experiments with mice were approved by the Institutional Animal Use and Care Committee of Boston Biomedical Research Institute and overseen by a veterinarian.

Histology and cell culture. Individual muscles were dissected, frozen immediately in 2-methylbutane chilled by liquid nitrogen, and 10-μm cryostat sections prepared from matching regions of muscles for comparison of different genotypes. Sections were stained with H&E. Cross-sectional areas of individual myofibers were determined using the NIH Image program. Myofibers with centrally located nuclei and total numbers of myofibers were counted manually. ATPase histochemistry (8) was used to identify fast and slow myofibers. For each measurement, muscle sections were prepared from the largest region of each muscle, and myofibers were measured and enumerated in multiple adjacent ×10 or ×20 microscope fields until the entire area of each muscle section was analyzed. Statistical significance was assessed by the appropriate χ^2 test, unpaired 2-tailed *t* test, Welch alternate *t* test, or nonparametric Mann-Whitney test using the InStat computer program (v2.03; GraphPad Software Inc.). For cell cultures, mononucleate cells were isolated from dissected muscles, enriched for satellite cells by density centrifugation, and cultured as in our previous work (8, 15). To assay experimentally induced apoptosis, cell cultures were treated with 100 nM staurosporine to induce apoptosis, and cells that died in response to staurosporine treatment were identified based on permeability to 5 μg/ml propidium iodide.

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