Activation of JNK1 contributes to dystrophic muscle pathogenesis

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Duchenne Muscular Dystrophy (DMD) originates from deleterious mutations in the dystrophin gene, with a complete loss of the protein product [1, 2]. Subsequently, the disease is manifested in severe striated muscle wasting and death in early adulthood [3]. Dystrophin provides a structural base for the assembly of an integral membrane protein complex [4]. As such, dystrophin deficiency leads to an altered mechanical integrity of the myofiber and a predisposition to contraction-induced damage [5–7]. However, the development of myofiber degeneration prior to an observed mechanical defect has been documented in various dystrophic models [8, 9]. Although activation of a detrimental signal transduction pathway has been suggested as a probable cause, a specific cellular cascade has yet to be defined. Here, it is shown that murine models of DMD displayed a muscle-specific activation of JNK1. Independent activation of JNK1 resulted in defects in myotube viability and integrity in vitro, similar to a dystrophic phenotype. In addition, direct muscle injection of an adenoviral construct containing the JNK1 inhibitory protein, JIP1, dramatically attenuated the progression of dystrophic myofiber destruction. Taken together, these results suggest that a JNK1-mediated signal cascade is a conserved feature of dystrophic muscle and contributes to the progression of the disease pathogenesis.

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Received: **16 November 2000**
Revised: **11 June 2001**
Accepted: **3 July 2001**

Published: **21 August 2001**

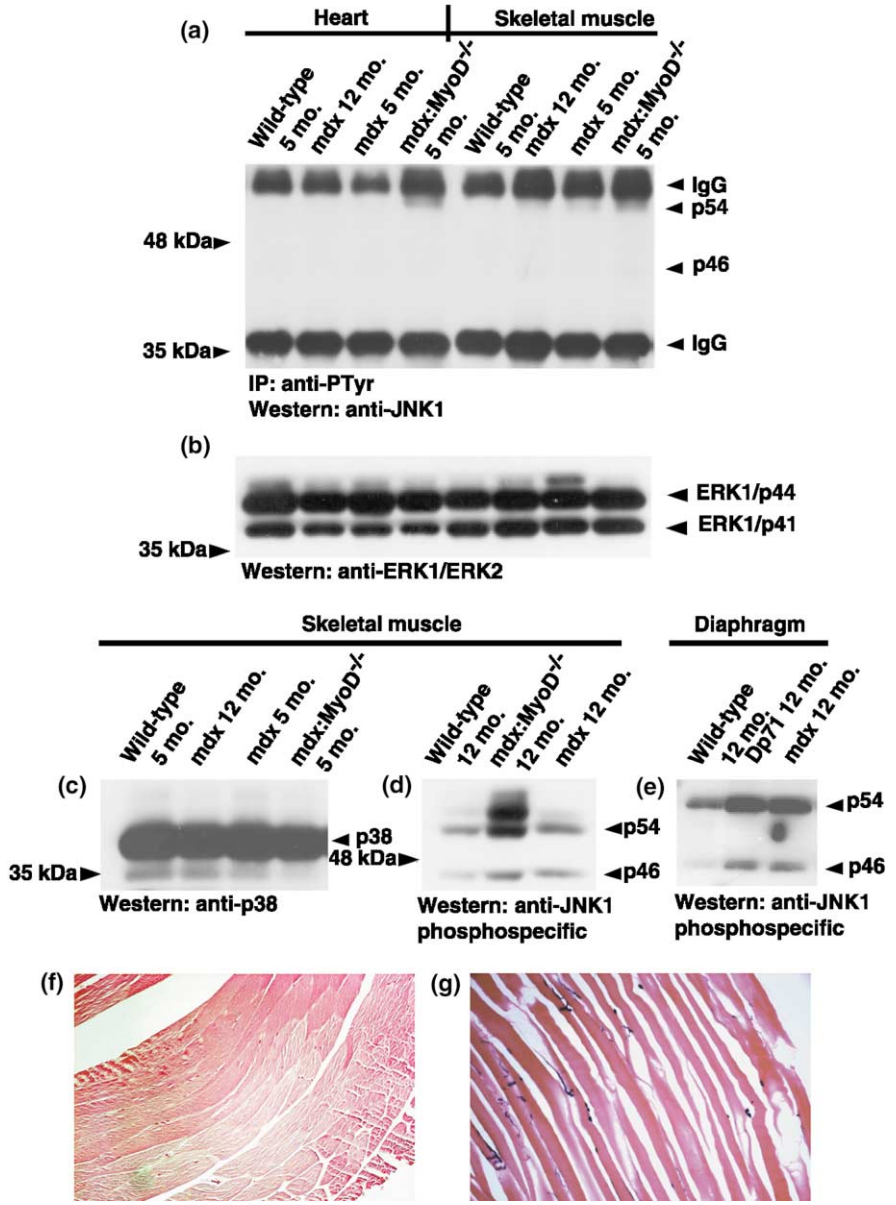
Current Biology 2001, 11:1278–1282
0960-9822/01/$ – see front matter

Results and discussion

Previously, we have shown that activation of MAPK pathways is associated with the evolving cellular milieu of the dystrophic myocardium [10, 11]. Therefore, MAPK phosphorylation/activity status was assessed in cardiac and skeletal muscle lysates collected from control mice and two murine dystrophic models, the mdx and mdx:MyoD$^{-/-}$ strains (Figure 1). The selection of both the mdx (murine genetic equivalent of DMD) and mdx:MyoD$^{-/-}$ strain provided a gradation in the dystrophic phenotype for these analyses (the loss of MyoD results in an inability to effectively repair damaged skeletal muscle; as such, the mdx:MyoD$^{-/-}$ strain displays a more pronounced dystrophy) [12]. Moreover, the cumulative damage observed in mdx:MyoD$^{-/-}$ skeletal muscle is also concurrent to a progressive cardiac dystrophy that is not present in the mdx mouse [10]. Altered phosphorylation of the stress-activated MAPK JNK1 (p54 isoform) was evident in immunoprecipitated (IPed) lysates derived from dystrophic muscle tissue, including cardiac and hindlimb skeletal muscle (Figure 1a); furthermore, JNK1 phosphorylation was not increased in tissues derived from the MyoD$^{-/-}$ parent strain [12] (data not shown). In addition, a longer exposure of the same IP/Western reveals evidence of the phosphorylated p46 isoform in the lysates with the greatest damage (similar to the pattern observed with p54). Unlike JNK1, the phosphorylation of ERK2 and p38 was not dramatically altered (Figure 1b,c).

Similarly, Western analysis with phosphospecific JNK1 revealed a substantial increase in JNK1 isoform phosphorylation (p54 > p46) from that of mdx:MyoD$^{-/-}$ lysates (Figure 1d). However, the phosphorylation status of JNK1 in mdx hindlimb muscle lysates was only marginally increased (Figure 1d), which may reflect the mild dystrophic phenotype of this murine model. Nevertheless, the diaphragm muscle of the mdx strain manifests the dystrophic phenotype and displays a dramatic increase in JNK1 phosphorylation (Figure 1e). In addition, lysates derived from diaphragm muscle of another dystrophic model, the Dp71 mouse [13], display similar levels of JNK1 phosphorylation. Finally, we examined the cellular specificity of JNK1 activation in dystrophic muscle. Immunostaining of murine dystrophic muscle sections with the phosphospecific JNK1 antibody confirmed JNK1 activation to be primarily within myonuclei and degenerating myofibers (Figure 1f,g). Together, these observations establish JNK1 activation as a conserved feature of dystrophic muscle pathogenesis, a feature that is directly proportional to the extent of muscle damage.

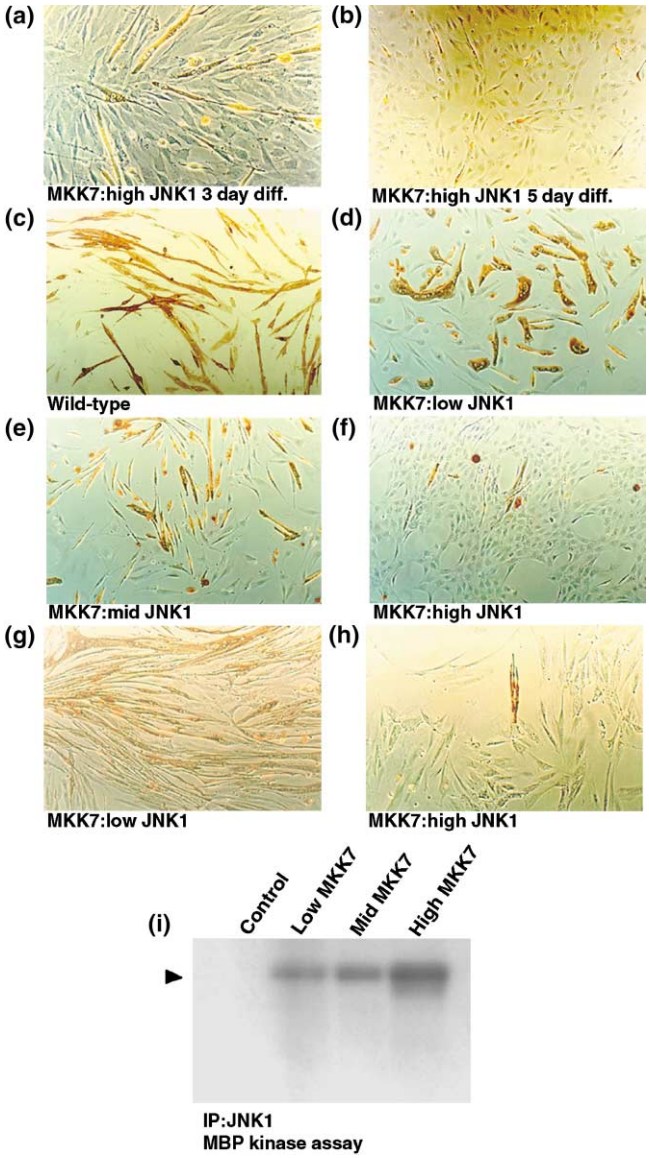
Figure 1



Constitutive activation of JNK1 in dystrophic heart and skeletal muscle. **(a)** Immunoblot analysis revealed that JNK1 phosphorylation was altered in mdx skeletal muscle and in both mdx:MyoD$^{-/-}$ heart and skeletal muscle. **(b)** Changes in ERK1/ERK2 mobility did not correspond to the changes observed with JNK1. Levels in both mdx:MyoD$^{-/-}$ heart and skeletal muscle remain low. **(c)** Western blot analysis of skeletal muscle using an anti-p38 antibody revealed no change in p38 migration/phosphorylation. **(d)** Immunoblots carried out with a phosphospecific anti-JNK1 antibody indicated a much higher level of activated JNK1 in mdx:MyoD$^{-/-}$ double mutants than in either wild-type or mdx skeletal muscle. **(e)** Western blots of mouse diaphragm muscle probed with a phosphospecific JNK1 antibody. Increased levels of JNK1 are evident in both Dp71 and mdx dystrophin mutants. **(f)** Wild-type tibialis muscle immunostained for phosphospecific JNK1 expression. No staining was evident. **(g)** Sections from mdx:MyoD$^{-/-}$ tibialis anterior muscle immunostained for phosphospecific JNK1 expression. Staining is evident in nuclei. The counterstain in both cases was eosin Y.

To determine whether elevated JNK1 activity induced a dystrophic muscle phenotype, C2C12 skeletal muscle myoblasts and H9C2 cardiac myoblasts were stably transfected with the JNK1-specific activating kinase, MKK7 [14, 15]. Stable transformants were then categorized by the level of MKK7 expression and JNK1 activity/phosphorylation. Initial assessments revealed that the growth kinetics and viability of all selected cell lines remained unchanged. Moreover, both myotube formation and differentiation were readily apparent within 3 days following serum withdrawal in all C2C12 and H9C2 clones examined (Figure 2a). Despite the neutral effect on replicating myoblasts and early myotube formation, MKK7 overexpression/JNK1 activation led to a decrease in myotube integrity (Figure 2a–h). Specifically, in C2C12 myoblast clones with high levels of MKK7 expression/JNK activity, the total number of myotubes decreased as the differentiation program proceeded. The MF-20-positive myocyte population declined from ~30% at 3 days postdifferentiation to less than 8% at 5 days postdifferentiation. This is in stark contrast to wild-type and low-expressing cell lines that increase from ~35% to >60% MF-20-positive cells over the same time course. This temporal decline in myotube integrity was preceded by an increased vacuolization and formation of picnotic nuclei (Figure 2a,b). Moreover, the severity of the myotube phenotype in both C2C12

Figure 2



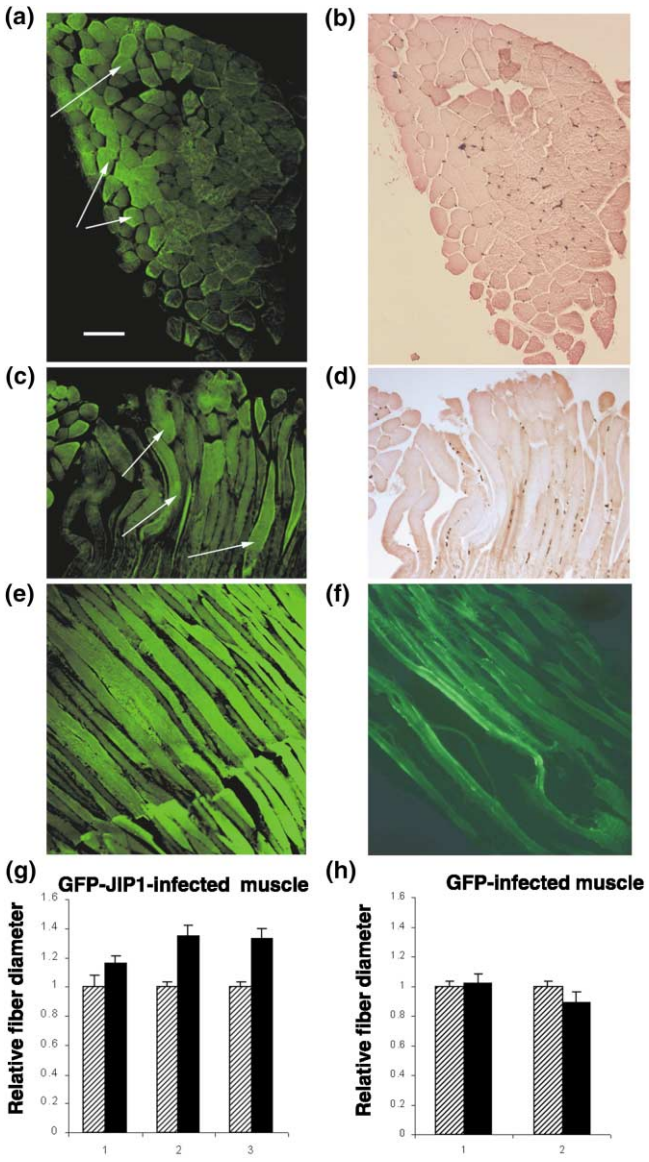
The effects of JNK1 activation in C2C12 skeletal myoblasts and H9C2 cardiac myoblasts stably transfected with the JNK1-specific activator MKK7. **(a,b)** Temporal decline in myotube number and viability during differentiation of MKK7:high JNK1 activity C2C12 cell lines ([a] 3 days differentiation; [b] 5 days differentiation). Disrupted myotubes and vacuolization are evident. **(c)** Wild-type C2C12 cells exhibited normal myotube formation (as demonstrated by immunostaining for striated muscle-specific myosin heavy chain [MF-20]). While some differentiation was evident with **(d)** low and **(e)** medium MKK7 expression, **(f)** high levels of MKK7 expression/JNK activity in C2C12 myocytes led to a decrease in myotube integrity and viability. **(g,h)** H9C2 cells likewise showed a similar reduction in myotube viability and MF-20 immunostaining with increased expression of MKK7. **(i)** Whole-cell lysates derived from C2C12 cells expressing differential levels of MKK7. Lysates were subjected to immunoprecipitation with JNK1 antibody, followed by an in vitro kinase assay using MBP as a substrate. The control was lysate-derived from untransfected, wild-type C2C12 cells. MBP phosphorylation increases as the level of expression increases.

and H9C2 clones was proportional to the level of JNK activation, i.e., myotube viability decreased as the level of MKK7 expression/JNK activity increased (Figure 2c–h; Figure 2i documents JNK1 activity in the various C2C12 stable cell lines overexpressing MKK7).

In order to assess the role of JNK1-mediated myofiber damage in vivo, we have employed the specific JNK inhibitor JIP1 (JNK interacting protein). JIP1 is a cytosol-restricted scaffolding protein that has been shown to prevent JNK1 activation via sequestration of JNK1 and its immediate upstream effector, MKK-7 [16]. JIP1 is highly specific for JNK1 and does not interact with other JNK family members or with other members of the JNK signal cascade [17]. An adenoviral construct containing JIP cDNA flanked by FLAG and GFP tags was injected into tibialis anterior muscles of 8-week-old mdx:MyoD$^{-/-}$ mice. Previous in vitro experiments using cultured sympathetic neurons showed that the adenoviral construct possessed excellent infectivity (G. Walsh, F. Miller, unpublished data). Furthermore, both JIP and GFP were detected on Western blots of lysates derived from infected cells (data not shown). Mice were sacrificed 3 weeks after viral introduction, and sectioned muscles were analyzed for the presence of GFP/JIP. In addition, GFP-positive myofiber diameters and overall health were compared to uninfected fibers within the same section and to uninjected contralateral controls. Animals injected with adenovirus encoding GFP alone served as an alternate control. In general, infected fibers appeared healthier and more robust than adjacent uninfected fibers (Figure 3a–d) or GFP controls (Figure 3f). While uninfected myofibers often appeared vacuolated and in various stages of degeneration, the integrity of JIP adenovirus-infected fibers was maintained, exhibiting a phenotype reminiscent of that observed in wild-type tissues. In addition, the diameters of JIP-infected myofibers were between 15% and 35% larger (paired t test, $p < 0.05$) than uninfected myofibers (Figure 3g). This was especially striking in areas of variable adenoviral infection, with robust JIP overexpressing fibers interspersed with much smaller, degenerate fibers (Figure 3a–e). Contralateral controls exhibited no fluorescence above background levels. No significant difference in fiber diameter was noted in animals injected with GFP alone (Figure 3h).

To delineate the mechanism by which JNK1 impaired myofiber integrity, we sought to identify corresponding targets for this kinase during the progression of dystrophic muscle pathogenesis. Preliminary results have shown that JNK1 activation in dystrophic muscle leads to an increased interaction with a member of the NF-AT transcription factor family NF-ATc1. In turn, this interaction results in the nuclear exclusion of the transcription factor in dystrophic muscle (See Supplementary material available with this article online). Of interest, NF-AT transcription

Figure 3



The effects of intramuscular administration of adenovirus containing JIP1 cDNA tagged with GFP. **(a)** Fluorescent microscopy image of a cross-section of the tibialis anterior muscle from a 12-week-old mdx:MyoD$^{-/-}$ mouse injected with adenovirus at 9 weeks . GFP fluorescence indicates infected myofibers (arrows). **(b)** A bright-field image of a cross-section counterstained with eosin Y. **(c)** A fluorescent microscopy image of a tibialis anterior longditudinal section from a 12-week-old mdx:MyoD$^{-/-}$ mouse injected with adenovirus at 9 weeks. Examples of infected fibers containing GFP/JIP are indicated (arrows). **(d)** A bright-field image of a cross-section counterstained with eosin Y. **(e)** A longitudinal section from mdx:MyoD$^{-/-}$ showing robust GFP/JIP1-positive fibers intermingled with degenerating, uninfected fibers. The scale bar in (a) represents 100 μm and is applicable to all images. **(f)** A longitudinal section of a tibialis muscle infected with adenovirus containing GFP alone. Fibers are tattered and appear damaged. **(g)** A comparison of the diameters of GFP/JIP1 adenovirus-infected and uninfected fibers. Data is normalized to the mean diameter of uninfected fibers. 1, 2, and 3 indicate data from separate injections. The bars represent the standard error. Overall, there was a significant increase in the diameters of infected fibers over uninfected fibers (paired t test, $p < 0.05$). **(h)** A comparison of the diameters of GFP adenovirus-infected and uninfected fibers. No significant difference in fiber diameters was seen.

factors have been described as key regulatory proteins for hypertrophic growth of both cardiac and skeletal muscle [18–20]. In addition, we have observed changes in gene expression coincident to JNK1 activation, which may accelerate the loss of myofiber integrity, i.e., JNK1-dependent upregulation of proapoptotic neuronal genes (See Supplementary material). Therefore, these results suggest that JNK1 activation may elicit myofiber destruction through multiple avenues.

Taken together, our results support a model in which the progression of dystrophic muscle pathogenesis is in part dependent upon JNK1 activation. The activation of JNK1 in dystrophic muscle may result from an intrinsic loss of the dystrophin protein and/or the cognate binding partners. Specifically, components of the dystrophin dystroglycan protein complex (DGC) have been shown to retain signaling molecules such as microtubule-associated serine/threonine kinase (MAST) and syntrophin-associated serine/threonine kinase (SAST) [21]. Therefore, the failure to retain these kinases in a membrane-bound DGC may provide a precipitating signal for the activation of JNK1 in a dystrophic muscle. Alternatively, JNK1 activation may originate as a secondary consequence of dystrophic myofiber destabilization. For example, disruption of the cytoskeleton (a hallmark of a dystrophic myofiber) in nonmuscle cells leads to JNK1 activation, followed by a requisite decline in cellular viability [22, 23]. Indeed, consideration of these issues raises the possibility that JNK1 activation may not be the sole arbiter of signal-associated myofiber destruction. Nevertheless, and despite the potential for signal promiscuity, the data suggest that JNK1 activation is a conserved characteristic of the dystrophic muscle phenotype.

Supplementary material

Supplementary material containing the materials and methods section and information concerning JNK1 targets and interactions as well as gene expression profiles associated with JNK1 activation is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgements

Special thanks to Jesse and John Davidson and Nancy Sinclair. We also thank Jim Woodgett for providing the MKK7 expression plasmid. This work was supported by grants to L.A.M. from the Muscular Dystrophy Association (USA), the Foundation for Cell and Gene Therapy (FCGT), and the Canadian Institutes of Health Research. S.M.K. was supported by a fellowship from the FCGT, and J.S. was supported by a studentship through the Ontario Graduate Student Science and Technology program.

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