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EXPRESSION OF INTERCELLULAR ADHESION MOLECULE-1 BY MYOFIBERS IN *mdx* MICE

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Abstract

Introduction—We investigated the extent to which intercellular adhesion molecule-1 (ICAM-1), a critical protein of the inflammatory response, is expressed in skeletal muscles of *mdx* mice (a murine model of Duchenne muscular dystrophy).

Methods—Muscles were collected from control and *mdx* mice at 2–24 weeks of age and analyzed for ICAM-1 expression by means of Western blot and immunofluorescence.

Results—Western blot revealed higher expression of ICAM-1 in *mdx* compared with control muscles through 24 weeks of age. In contrast to control muscles, ICAM-1 was expressed on the membrane of damaged, regenerating, and normal myofibers of *mdx* mice. CD11b⁺ myeloid cells also expressed ICAM-1 in *mdx* muscles, and CD11b⁺ cells were closely associated with the membrane of myofibers expressing ICAM-1.

Conclusions—These findings support a paradigm in which ICAM-1 and its localization to myofibers in muscles of *mdx* mice contributes to the dystrophic pathology.

Keywords

$\beta 2$ integrins; Duchenne muscular dystrophy; macrophages; muscle inflammation; neutrophils

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular disease caused by a mutation in the gene for dystrophin. While a mutation in the gene for dystrophin is the proximal cause of DMD, the inflammatory response contributes to the onset and progression of the muscle pathology. Specifically, myeloid cells (e.g., neutrophils and macrophages), free radicals, and cytokines (e.g., IFN- γ , TNF- α , and TGF- $\beta 1$) of the inflammatory response induce myofiber damage, inhibit regeneration, and promote fibrosis in dystrophin deficient muscles of *mdx* mice,^{1–8} a murine model of DMD.⁹ Mechanisms through which the inflammatory response exacerbates the dystrophic pathology are not well understood.

Myeloid cell adhesion to membrane structures of cells and components of the extracellular matrix serve as mechanisms for initiating production of potentially deleterious free radicals and cytokines.¹⁰ This adhesion is facilitated by $\beta 2$ integrins, which are leukocyte-specific heterodimeric glycoproteins composed of a common β subunit (CD18) and 1 of 4 α subunits (CD11a, CD11b, CD11c, or CD11d).^{10,11} We have previously demonstrated that $\beta 2$ integrins serve as a mechanism for neutrophil accumulation, oxidative damage, and structural and functional deficits after contraction-induced muscle injury,¹² as well as a mechanism through which adhesion-induced reactive oxygen species (ROS) production from myeloid cells damages cultured skeletal muscle cells.^{13,14} In *mdx* mice, CD11b contributes to the muscle pathology through its ability to bind to fibrinogen and initiate cytokine production from myeloid cells.^{2,4} The extent to which other ligands for $\beta 2$ integrins are expressed in muscles of *mdx* mice has yet to be established.

A major ligand for CD11a and CD11b is inter-cellular adhesion molecule-1 (ICAM-1; CD54), a member of the immunoglobulin superfamily of adhesion molecules.¹¹ Vascular endothelial cells and leukocytes normally express ICAM-1 at low levels,¹¹ while skeletal muscle cells *in vitro* and *in vivo* do not constitutively express ICAM-1.^{15–20} Endothelial cell expression of ICAM-1 serves as a mechanism for $\beta 2$ integrin-mediated entry of myeloid cells into skeletal muscle,^{11,21} and ICAM-1 expression by myeloid cells facilitates their homotypic adhesion^{22,23} and production of ROS and cytokines.^{24–26} Importantly, endothelial cell, leukocyte, and myofiber expression of ICAM-1 has been observed in muscle biopsies obtained from patients with inflammatory myopathies^{15,16} and DMD.¹⁷

The purpose of this study was to examine ICAM-1 expression and its cellular localization in skeletal muscles of *mdx* mice when signs of disease progression are apparent. In particular, we determined the extent to which ICAM-1 was expressed by damaged, regenerating, and normal myofibers of *mdx* mice to gain insight into the potential contribution of ICAM-1 to the pathophysiology of DMD. We hypothesized that ICAM-1 expression by myofibers in *mdx* muscles is temporally associated with pathological features of DMD.

MATERIALS AND METHODS

Mice

The *mdx* mice (C57BL/10 ScSn DMD^{mdx}; Jackson Laboratory) used in this study were bred in the animal facilities at The Ohio State University College of Medicine, and control mice (C57BL/6; Jackson Laboratory) were bred in an animal facility at the University of Toledo. Mice were housed on a 12-h light-dark cycle and fed standard laboratory chow and water *ad libitum*. Hindlimb muscles (gastrocnemius and tibialis anterior) were collected from anesthetized mice at ages 2, 3, 4, 10, and 24 weeks and frozen for subsequent analysis. Experimental procedures were approved by the institutional animal care and use committees at The Ohio State University and University of Toledo.

Western Blotting

Skeletal muscles were homogenized in reducing sample buffer (2% sodium dodecyl sulfate [SDS], 1.5% dithiothreitol, 80 mM Tris-HCl, and 10% glycerol) containing protease

inhibitors [1 mM ethylenediamine tetraacetic acid, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 11 mM 4-(2-aminoethyl) benzenesulfonyl fluoride] with a bead homogenizer (Tissue Lyser; Qiagen). Homogenates were centrifuged ($5,000 \times g$, 4°C, 10 min), and the amount of protein in supernatants was determined.²⁷

Samples were boiled and separated on 10% SDS-polyacrylamide gels (15 μ g of protein per lane). Control and *mdx* samples were loaded on the same gel. Proteins within the gel were transferred for 1 h to PVDF-FL membranes (Millipore) in Towbin transfer buffer containing 10% methanol using a semi-dry (20 volts) protocol. Membranes were blocked (50% Odyssey® Blocking buffer in Tris-buffered saline; LI-COR Biosciences) and incubated overnight at 4°C with an antibody that recognizes ICAM-1 (R&D Systems Cat # AF796; 1:500) or GAPDH (loading control; Cell Signaling Cat# 2118; 1:7,500). The relative abundance of ICAM-1 was quantified using the Odyssey® infrared detection system (LI-COR Biosciences).

Immunofluorescence

Acetone fixed transverse sections (10 μ m) were treated with blocking buffer (3% bovine serum albumin, 0.05% Tween-20, 0.2% gelatin in phosphate buffered saline), washed, and incubated with 1 or more of the following anti-mouse antibodies: anti-ICAM-1 (1:20; R&D Systems product # AF796), anti-CD31 (1:50; BD Pharmingen product # 550274), anti-CD11b (1:50; BD Pharmingen product # 550282), and anti-IgG (1:200; Jackson ImmunoResearch Laboratories product #515-165-003). Sections serving as negative controls received phosphate buffered saline instead of primary antibody. Sections were then incubated with a fluorescein isothiocyanate (FITC) or cyanine 3 (Cy3) conjugated secondary antibody. Nuclei were stained with 4',6-Diamidino-2-phenylindole (DAPI) by mounting muscle sections in Fluoromount-G (SouthernBiotech). Muscle sections were viewed using epifluorescence microscopy (Olympus IX70) and imaged using a CCD camera (RT KE SPOT™; Diagnostic Instruments).

Damaged, Regenerating, and Normal Myofibers

Damaged myofibers in *mdx* muscles were defined by the presence of murine IgG and/or cells in the myofiber sarcoplasm, suggestive of membrane lesions. Regenerating myofibers were defined as those with central nucleation. Myofibers that showed no signs of damage, or central nucleation were classified as normal.

The number of damaged, regenerating, and normal myofibers in an entire section of a muscle was counted manually and then expressed relative to the total number of myofibers within the section. We also counted the number of damaged, regenerating, and normal myofibers that showed membrane and cytoplasmic localization of ICAM-1 (ICAM-1+). These ICAM-1+ myofibers were expressed relative to the total number of ICAM-1+ myofibers within the section. On average, ~4,000 myofibers per muscle section were analyzed for signs of myofiber damage, regeneration, and ICAM-1 expression.

Histology

Transverse sections (10 μm) of muscles were stained with Masson trichrome using a kit according to the manufacture's protocol (IMEB Inc.), which stains myofibers red and collagen blue. Collagen accumulation within *mdx* muscles was qualitatively assessed as a measure of fibrosis.

Statistical Analyses

Western blot data were analyzed with a 2-way analysis of variance using mouse strain (*mdx* and control) and age (2, 3, 4, 10, and 24 weeks) as grouping factors (SigmaStat; Systat Inc). The percentage of ICAM-1+ myofibers in *mdx* muscles that were classified as normal, damaged, or regenerating was statistical analyzed using a 1-way analysis of variance. The percentage of damaged and regenerating myofibers in *mdx* muscles was not analyzed statistically, as it served as a descriptive measure of the muscle pathology.

RESULTS

The relative abundance of ICAM-1 was higher ($P < 0.05$) in *mdx* muscles compared with control muscles through 24 weeks of age (Fig. 1). ICAM-1 expression was similar between the strains of mice at age 2 weeks and 2- to 6-fold higher in *mdx* compared with control mice at ages 3–24 weeks. At age 3 weeks, elevated levels of ICAM-1 in *mdx* muscles preceded overt signs of myofiber damage, which was most prevalent at age 4 weeks (Fig. 2). Furthermore, ICAM-1 expression remained elevated in muscles of *mdx* mice at age 10 and 24 weeks, when signs of muscle regeneration and fibrosis were the prevailing pathological feature. Little to no signs of fibrosis were observed in muscle sections from 3 and 4-week-old *mdx* mice (images not shown).

Immunofluorescence was performed to determine the cellular localization of ICAM-1. As expected, CD31+ endothelial cells expressed ICAM-1 in both control and *mdx* muscles (Fig. 3A and B). Myofibers in control muscles did not express ICAM-1 (Fig. 3A), which is consistent with prior studies.^{15–17,20} In contrast, ICAM-1+ myofibers were found in muscles of *mdx* mice at ages 3–24 weeks (Fig. 3C–F). On average, ~100, 20, 30, and 50 ICAM-1+ myofibers per section of *mdx* muscle were found at age 3, 4, 10, and 24 weeks, respectively. Sections of control and *mdx* muscles treated with only a FITC-conjugated secondary antibody showed no labeling. Importantly, ICAM-1 was expressed by damaged (arrowhead in Fig. 3), regenerating (broken arrow in Fig. 3), and normal (arrow in Fig. 3) myofibers in *mdx* muscles.

To further characterize myofiber expression of ICAM-1, we counted the number of ICAM-1+ myofibers in *mdx* muscles that showed overt signs of damage and regeneration, and expressed them relative to the total number of ICAM-1+ myofibers within the muscle section (Fig. 4). At ages 3 and 4 weeks, 60–70% of the ICAM-1+ myofibers showed signs of damage, but only 4% of the ICAM-1+ myofibers were regenerating myofibers. The percentage of ICAM-1+ myofibers that were damaged did not change statistically ($P > 0.05$) with advancing age, while the percentage of ICAM-1+ myofibers that were regenerating was higher ($P < 0.05$) at age 24 weeks compared with age 3 weeks. Of interest, we found

ICAM-1+ regenerating myofibers that were damaged, particularly at age 10 weeks (Fig. 3E). The percentage of ICAM-1+ myofibers in *mdx* muscles that exhibited no overt signs of damage or regeneration (i.e., normal myofibers) remained relatively constant throughout age 24 weeks.

To gain insight into the possibility that interaction between CD11b+ cells and ICAM-1+ myofibers contributes to the dystrophic pathology, we performed double labeling for CD11b and ICAM-1. Numerous CD11b+ cells were found in *mdx* muscles, and many of them expressed ICAM-1 (Fig. 5). These observations are in agreement with prior work that demonstrated myeloid cell accumulation in dystrophic muscles,¹⁻⁸ as well as the established expression of ICAM-1 by myeloid cells.¹¹ Importantly, CD11b+ cells that were ICAM-1+ or ICAM-1- were associated closely with the membrane of ICAM-1+ myofibers, and CD11b+ cells were found in the sarcoplasm of myofibers. In the latter case, it was difficult to discern whether the ICAM-1 in myofibers invaded by CD11b+ cells represents myofiber or myeloid cell expression of ICAM-1 (Fig. 5C).

DISCUSSION

Mechanisms through which the inflammatory response contributes to the onset and progression of DMD have yet to be elucidated. Prior work has focused on the contribution of macrophages and cytokines to pathological features of DMD in *mdx* mice. Little scientific attention has been directed toward determining the extent to which adhesion molecules of the inflammatory response contribute to the dystrophic pathology. Findings from this study begin to address this deficiency by demonstrating that ICAM-1, an important adhesion molecule of the inflammatory response,¹¹ is elevated in *mdx* muscles before overt signs of myofiber damage and remains elevated during the degenerative, regenerative, and fibrotic phases of the pathology. Importantly, damaged, regenerating, and normal myofibers in *mdx* mice expressed ICAM-1. ICAM-1 was also expressed by myeloid cells in *mdx* muscles, and myeloid cells were closely associated with the membrane of ICAM-1+ myofibers, as well as within necrotic myofibers. Altogether, our findings support a paradigm in which ICAM-1 expression contributes to the onset and progression of the muscle pathology in *mdx* mice.

Prior investigators have examined ICAM-1 expression in muscles of patients with inflammatory myopathies^{15,16} and DMD.¹⁷ In patients with DMD, ICAM-1 was expressed by necrotic and regenerating myofibers.¹⁷ We have extended these findings by demonstrating that myofibers in *mdx* muscles express ICAM-1 and that their expression of ICAM-1 is not specific to myofiber damage or regeneration. Rather, the morphological characteristic of myofibers that expressed ICAM-1 reflected the prevailing pathological feature. Specifically, ICAM-1 was expressed by damaged and regenerating myofibers when signs of myofiber damage and regeneration, respectively were most prevalent. The finding that ICAM-1 is expressed by myofibers in *mdx* muscles is important, as it establishes a murine model through which the contribution of ICAM-1 to the dystrophic pathology can be revealed using pharmacological and genetic approaches.

Mechanisms for increased expression of ICAM-1 in *mdx* muscles remain to be determined. ICAM-1 expression is regulated primarily at the level of gene transcription, and the ICAM-1

promoter contains binding sites for several transcription factors (e.g., nuclear transcription factor- κ B, NF κ B) that become active in response to cytokines and ROS.^{28,29} Indeed, increased expression of ICAM-1 by endothelial and leukocytes,¹¹ and induced expression of ICAM-1 by cultured skeletal muscle cells^{18–20} occurs after treatment with cytokines (e.g., IFN- γ and TNF- α) or ROS. As several cytokines that regulate ICAM-1 expression and NF κ B activity are elevated in *mdx* muscles,^{1–3,5,29,30} we speculate that cytokines in the microenvironment of *mdx* muscles increased ICAM-1 expression through a mechanism involving NF κ B signaling.

The function of ICAM-1 expressed by myofibers in *mdx* muscles remains to be determined. ICAM-1 is a membrane glycoprotein consisting of 5 extracellular domains, a transmembrane segment, and a short cytoplasmic domain.¹¹ The extracellular domains of ICAM-1 facilitate cell-to-cell communication by serving as a point of attachment for leukocytes expressing CD11a (e.g., lymphocytes) and CD11b (e.g., myeloid cells). Thus, the expression of ICAM-1 by myofibers in dystrophin deficient muscles likely serves as a means through which leukocytes adhere to them by means of a β 2 integrin dependent mechanism. This interpretation is consistent with the findings of CD11a⁺¹⁷ and CD11b⁺ cells (present study) closely associating with the membrane of ICAM-1⁺ myofibers, as well as their accumulation in the sarcoplasm of myofibers in dystrophin deficient muscles. As ligation of ICAM-1 by β 2 integrins facilitates myeloid cell adhesion and production of potentially deleterious cytokines and ROS,^{24–26} we speculate that ICAM-1 expression by myofibers in dystrophin deficient muscles serves as a means through which myeloid cells adhere to myofibers and subsequently exacerbate the muscle pathology. This interpretation is consistent with prior studies that reported myeloid cells and β 2 integrins exacerbate the dystrophic pathology.^{1–8} In contrast, macrophages, β 2 integrins, and ICAM-1 in nondiseased muscle produce beneficial outcomes after injury or mechanical loading (muscle overload).^{20,31–35} As the phenotype of myeloid cells is influenced by both cytokines^{36,37} and their adhesion,^{10,22–26} differences in the local environment of diseased and nondiseased muscles likely determines the extent to which myeloid cell adhesion to myofibers produces deleterious or beneficial outcomes in skeletal muscle.

Our findings establish the relevance of ICAM-1 in the pathophysiology of DMD by demonstrating that ICAM-1 is highly expressed and localized to myofibers in *mdx* muscles during the degenerative, regenerative, and fibrotic phases of the pathology. Further work is needed to determine the extent to which ICAM-1 expression contributes to the dystrophic pathology. Elucidating the function of ICAM-1 in dystrophy deficient muscles will help define novel therapeutic targets to slow the timescale of DMD and extend patient survival.

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Abbreviations

DMD Duchenne muscular dystrophy

GAPDH	glyceral-dehydes-3-phosphate dehydrogenase
ICAM-1	intercellular adhesion molecule-1
IFN-γ	γ interferon
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
TNF-α	tumor necrosis factor- α
TGF-β1	transforming growth factor β 1

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FIGURE 1.

ICAM-1 expression. **(A)** Representative western blot of ICAM-1 (110 kDa) and GAPDH (loading control) in control and *mdx* mice at ages 2, 3, 4, 10, and 24 weeks (15 μ g of protein/lane). **(B)** Quantitative analysis of ICAM-1 protein ($n = 4-5$ /group). #, significantly elevated in *mdx* compared with control muscles through 24 weeks of age. Data are reported as mean $\pm$ SEM.

FIGURE 2.

Muscle pathology in *mdx* mice. Damaged myofibers were identified by the presence of murine IgG (red; arrow in **A**) and/or accumulation of nuclei/cells (blue; arrow in **B**) in the sarcoplasm of myofibers. Myofibers with central nucleation were classified as regenerating (arrowhead in panels A and B). Scale bar = 25 μ m. (**C**) Quantitative analysis of myofiber damage and regeneration. The number of damaged or regenerating myofibers is expressed relative to the total number of myofibers within a section ($n = 2-3$ muscles/age; mean $\pm$ SEM). On average, ~4,000 myofibers per muscle section were analyzed. Muscle sections from *mdx* mice stained with Masson trichrome. Muscle fibrosis is indicated by accumulation of collagen (blue) within the section, which was most evident in 10- (**D**) and 24- (**E**) week-old *mdx* mice. Scale bar = 25 μ m.

FIGURE 3.

Localization of ICAM-1 in *mdx* muscles. Endothelial cells in muscle sections were identified by CD31 expression (red). The majority of CD31+ cells were found to express ICAM-1 (green) in control (**A**) and *mdx* (**B**) muscles. In contrast to control muscles, myofibers in muscles of 3- to 24-week-old *mdx* mice showed strong membrane and weak sarcoplasmic localization of ICAM-1 (green) (**C–F**). To characterize myofiber expression of ICAM-1, we assessed myofiber damage by the accumulation of IgG (red) and nuclei/cells (blue) in myofiber sarcoplasm. Regenerating myofibers were defined as those with central nucleation. ICAM-1 was expressed by damaged (arrowhead in panels C, D, and F), regenerating (broken arrow in panels D and F), and normal (arrow in panels C, E, and F) myofibers in *mdx* muscles. Damaged, regenerating myofibers also expressed ICAM-1 (arrowhead and broken arrow in panel F). Scale bar = 25 μ m.

FIGURE 4.

Characterization of myofibers expressing ICAM-1 in *mdx* muscles. The number of damaged, regenerating, and normal myofibers that showed membrane and sarcoplasmic localization of ICAM-1 was counted (ICAM-1+) and then expressed as a percentage of the total number of ICAM-1+ myofibers within the section. Plotted values represent the mean of 2–3 muscles per age of *mdx* mice.

FIGURE 5.

CD11b and ICAM-1 localization in *mdx* muscles. Myeloid cells were identified by their expression of CD11b (red). Many of the CD11b+ cells in *mdx* muscles expressed ICAM-1 (green). (A,B) CD11b+ cells that were ICAM-1+ or ICAM-1– were associated closely with the membrane of ICAM-1+ myofibers (arrow in A and B), including regenerating myofibers (broken arrow in B). (C) CD11b+ cells were also found in the sarcoplasm of myofibers (arrowhead), which made it difficult to discern whether the ICAM-1 in sarcoplasm of such myofibers reflects myofiber or myeloid cell expression of ICAM-1.