Satellite cells and utrophin are not directly correlated with the degree of skeletal muscle damage in \textit{mdx} mice

Akira Yamane, Satonari Akutsu, Thomas G. H. Diekwisch, and Ryoichi Matsuda. Satellite cells and utrophin are not directly correlated with the degree of skeletal muscle damage in \textit{mdx} mice. \textit{Am J Physiol Cell Physiol} 289: C42–C48, 2005. First published February 9, 2005; doi:10.1152/ajpcell.00577.2004.—To determine whether muscle satellite cells and utrophin are correlated with the degree of damage in \textit{mdx} skeletal muscles, we measured the area of the degenerative region as an indicator of myofiber degeneration in the masseter, gastrocnemius, soleus, and diaphragm muscles of \textit{mdx} mice. Furthermore, we analyzed the expression levels of the paired box homeotic gene 7 (\textit{pax7}), m-cadherin (the makers of muscle satellite cells), and utrophin mRNA. We also investigated the immunolocalization of m-cadherin and utrophin proteins in the muscles of normal C57BL/10J (B10) and \textit{mdx} mice. The expression level of \textit{pax7} mRNA and the percentage of m-cadherin-positive cells among the total number of cell nuclei in the muscle tissues in all four muscles studied were greater in the \textit{mdx} mice than in the B10 mice. However, there was no significant correlation between muscle damage and expression level for \textit{pax7} mRNA ($R = -0.140$), nor was there a correlation between muscle damage and the percentage of satellite cells among the total number of cell nuclei ($R = -0.411$) in the \textit{mdx} mice. The expression level of utrophin mRNA and the intensity of immunostaining for utrophin in all four muscles studied were greater in the \textit{mdx} mice than in the B10 mice. However, there also was not a significant correlation between muscle damage and expression level of utrophin mRNA ($R = 0.231$) in the \textit{mdx} mice, although upregulated utrophin was incorporated into the sarcolemma. These results suggest that satellite cells and utrophin are not directly correlated with the degree of skeletal muscle damage in \textit{mdx} mice.

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In the present study, to determine whether muscle satellite cells and the expression of utrophin are correlated with the degree of damage in \textit{mdx} skeletal muscles, we measured the area of the degenerative region relative to the total muscle area, an indicator of myofiber degeneration, in the masseter, gastrocnemius, soleus, and diaphragm muscles of the \textit{mdx} mouse. Furthermore, we analyzed the mRNA expression levels of the paired box homeotic gene 7 (\textit{pax7}), m-cadherin (maker of muscle satellite cells), and utrophin and investigated the immunolocalization of m-cadherin and utrophin in the muscles of both normal and \textit{mdx} mice.

**MATERIALS AND METHODS**

\textit{Experimental animals.} Dystrophic \textit{mdx} and control C57BL/10J (B10) mice, obtained from the Central Animal Research Laboratory (Kanagawa, Japan), were used throughout the present study. All mice were housed under a 12-h light/dark cycle with free access to water and standard chow. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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were maintained on a 12:12-h light-dark cycle, fed a pellet diet (CE-2; Clea Japan, Tokyo, Japan), and allowed access to tap water ad libitum. All experimental protocols concerning animal handling were reviewed and approved by the Institutional Animal Care Committee of the Tsurumi University School of Dental Medicine.

Histological analysis of degenerative area. Six mdx and six control B10 mice, aged 6 wk, were killed by cervical dislocation under ether anesthesia. The body weights of the mdx and B10 mice were 17.2 ± 1.9 g and 18.9 ± 1.5 g (mean ± SD), respectively. Whole portions of the left masseter, gastrocnemius, soleus, and diaphragm muscles were removed and fixed in Bouin’s fixative for 1 h at 4°C. After being washed in phosphate-buffered saline (PBS), the sections were immersed in a graduated series of sucrose solutions (−20–40% wt/vol) in PBS at 4°C, embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, IN), and frozen. Middle portions of the muscles were cut at 10-µm thickness using a cryostat and air dried for 1 h at room temperature. The sections were stained with hematoxylin and eosin and observed under a light microscope. The total and degenerative areas of the muscle sections were measured using an image analyzer (Luzex3U; Nikon, Tokyo, Japan). The degenerative area was normalized to the total area and expressed as a percentage of the total area of the muscles.

Immunohistochemistry for m-cadherin and utrophin. To detect the satellite cells in the mdx and B10 muscles, the cryosections of the muscles were immunostained for a goat antibody against m-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (31). The number of m-cadherin-positive cells in the periphery of myofibers and in the degenerative area was counted as satellite cells in a total of 10 rectangular areas of 140 × 130 µm² on several sections obtained from each mouse (21). The 10 rectangular areas, which did not overlap and were uniformly distributed on the sectional area of muscles, were selected throughout the several sections and composed ~5–20% of the total sectional area of the B10 and mdx muscles. The number of total cell nuclei in muscle tissues was also counted, and the percentage of m-cadherin-positive cells to the total number of cell nuclei in the muscle tissues was calculated. The percentage values in the 10 rectangular areas were averaged to obtain the mean value for each mouse. This mean value was further averaged to obtain the mean values for six mdx and six B10 mice. To analyze the change in the expression of utrophin, immunostaining for utrophin (Santa Cruz Biotechnology) was performed using a goat antibody against utrophin (Santa Cruz Biotechnology). For control staining, the primary antibody was replaced with PBS or normal goat IgG.

RNA extraction, reverse transcription, and competitive polymerase chain reaction amplification. Six mdx and six B10 mice, aged 6 wk, were killed by cervical dislocation while they were under ether anesthesia. The body weights of the mdx and B10 mice were 19.8 ± 0.8 g and 24.3 ± 0.5 g (means ± SD), respectively. Whole portions of the left masseter, gastrocnemius, soleus, and diaphragm muscles were removed, immediately frozen, and stored at −80°C until use.

Total RNA extraction, reverse transcription, and competitive polymerase chain reaction (PCR) amplification were performed as previous.
ously described (34, 36). Briefly, total RNA extraction was performed according to the manufacturer’s specifications (rapid total RNA isolation kit; 5 Prime-3 Prime, Boulder, CO). The RNA was treated with 2 U of ribonuclease-free deoxyribonuclease I (Life Technologies, Gaithersburg, MD) and was then reverse transcribed with 200 U of reverse transcriptase (SuperScript II; Life Technologies).

In the conventional PCR technique, a small difference in the starting amount of target DNA can result in a large change in the yield of the final product, owing to the exponential nature of the PCR reaction. A plateau effect after many cycles can lead to an inaccurate estimation of final product yield. Furthermore, because the PCR amplification depends on the reaction efficiency, small changes in efficiency can lead to major differences in the final product yield. To overcome these problems, the competitor (an internal standard), which has the same primer sequences as those of the target DNA at the 5’ and 3’ ends, was amplified simultaneously with the target (11, 25, 34, 36). Competitors for the competitive PCR amplification were constructed according to the manufacturer’s instructions (Competitive DNA Construction Kit; Takara, Shiga, Japan). The sequences of primers for pax7, m-cadherin, and utrophin were as follows: pax7, FW, 5’-CCACAGCTTCTCCAGCTACTG-3’ and BW, 5’-CCTGCCGCTGAGGTGCCAAG-3’ (29); m-cadherin, FW, 5’-ATGGCTGCACTGCACATCG-3’ and BW, 5’-CCATATGCCTGCCAGC-3’ (14); utrophin, FW, 5’-AAACTCTTATCATCATATCA-3’ and BW, 5’-CTCACGTGCCGCTTCTCCT-3’ (9). Those for S16 were identical to those used in a previous report (18).

The amplification products were isolated by performing electrophoresis with an agarose gel containing ethidium bromide. The fluorescence image analysis data of electrophoretic bands shown in Fig. 1 shows the standard curve for m-cadherin calculated using the log-log formula derived from a standard curve for each target gene. The logarithmic value of the fluorescence intensity ratio was used to normalize the quantity of each target gene to the quantity of S16 (ribosomal protein). The resulting ratio value in each sample was expressed as a percentage of the mean value for the B10 masseter muscle. Because each percentage value relative to the mean value for the B10 masseter muscle (% of B10 masseter value) was an arbitrary unit, it was used in the scatterplots shown in Figs. 3–5, although the scatterplots contain no data regarding the B10 muscles.

**Statistical analyses.** The Tukey-Kramer method was used to compare multiple combinations of two muscles among the four mdx muscles. Student’s t-test was used to compare single combinations of the same types of muscles between the B10 and mdx mice. Regression analysis was used to examine the correlations in the mdx mice between the degenerative areas of the skeletal muscles and the expression levels for pax7 or utrophin mRNA and between the degenerative areas of the skeletal muscles and the percentages of m-cadherin-positive cells to the total number of cell nuclei in the muscle tissues. Differences were considered significant at P < 0.05.

**RESULTS**

To detect the degenerative area of the mdx muscle, cryosections from the middle portions of the muscles were stained with hematoxylin and eosin (Fig. 2, A–D). In the mdx gastrocnemius and diaphragm muscles, histopathological changes such as myofiber degeneration (Fig. 2, B and D) were observed. By contrast, none of the muscles of the control B10 mice showed such histopathological changes (data not shown). Figure 2E shows the degenerative area relative to the total cross-sectional area of the mdx muscles. The degenerative areas of the diaphragm and gastrocnemius muscles were 11.7 ± 3.6% and 8.5 ± 3.7% of the total muscle areas, respectively. These areas were significantly greater than those of the masseter (2.8 ± 1.6%) and soleus muscles (2.4 ± 1.5%) (P < 0.01 to gastrocnemius and P < 0.001 to diaphragm).

To determine whether the activity and pool size of muscle satellite cells are correlated with the degree of damage in mdx

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**Fig. 3.** A: typical example of gel electrophoretic pattern for paired box homeotic gene 7 (pax7)-competitive RT-PCR products of the masseter, gastrocnemius, soleus, and diaphragm muscles obtained from control C57BL/10J (B10) and mdx mice. The target gene is shown in the bottom bands, while the competitor is shown in the top band. B: mRNA expression levels for pax7 in the masseter, gastrocnemius, soleus, and diaphragm muscles obtained from B10 and mdx mice. Each column and vertical bar represent the mean ± SD of six B10 or mdx mice. The y-axis is expressed as a percentage of the mean value of the B10 masseter muscle set at 100. *P < 0.05 and **P < 0.01, significant differences between B10 and mdx mice. Φφφ < 0.01, significant difference among mdx muscles. C: scatterplot and regression line between degenerative areas of skeletal muscles and expression levels of pax7 in mdx mice.
skeletal muscles, we analyzed the expression of *pax7* (Fig. 3) and m-cadherin (Fig. 4), which are both markers for muscle satellite cells. Figure 3A shows a typical gel electrophoretic pattern of *pax7*-competitive PCR products for the masseter, gastrocnemius, soleus, and diaphragm muscles of the B10 mice. The fluorescence intensities of the *pax7* bands (bottom band) and of its respective competitor bands (top band) were measured using an image analyzer. Image analysis of the PCR bands indicated that in all muscles studied except the gastrocnemius muscle, the expression levels of *pax7* mRNA were significantly higher in the *mdx* mice than in the B10 mice (*P* < 0.05 and *P* < 0.01) (Fig. 3B). In the *mdx* mice, the expression level for *pax7* mRNA in the masseter muscle was more than sixfold the levels in the soleus and diaphragm muscles (*P* < 0.01) but not significantly different from that in the gastrocnemius muscle. Figure 3C shows a scatterplot and a regression line between the degenerative areas of the skeletal muscles and the expression levels of *pax7* in the *mdx* mice. The correlation coefficient was −0.140, which was not significantly different from zero.

In all muscles studied, the mean values of the expression levels for m-cadherin mRNA were higher in the *mdx* mice than in the B10 mice; only the 2.4-fold increase in the diaphragm muscle was statistically significant (Fig. 4A). In the *mdx* mice, no significant difference in the expression levels for m-cadherin mRNA was found among the muscles. Because m-cadherin is reportedly expressed in neural cells within muscle tissues (19), we also investigated the immunolocalization of m-cadherin. The number of m-cadherin-positive cells in the periphery of myofibers (arrow in Fig. 4B) and in the degenerative area (arrows in Fig. 4C) was counted as satellite cells. M-cadherin-positive cells in the areas interspaced among myofibers (arrows in Fig. 4D) were excluded. In all muscles studied except the gastrocnemius muscle, the ratio of satellite cells to the total number of cell nuclei in the muscle tissues was significantly higher in the *mdx* mice than in the B10 mice (*P* <
0.05 and \( P < 0.001 \) (Fig. 4E). In the mdx mice, the percentage of satellite cells to the total number of cell nuclei in the muscle tissues in the masseter muscle was \(-2.7\) to 5.4-fold those in the other three muscle types \( (P < 0.01) \). The correlation coefficient between the degenerative areas of the skeletal muscles and the percentage of m-cadherin-positive cells to the total number of cell nuclei in the muscle tissues in the mdx mice was \(-0.411 \), which was not significantly different from zero (Fig. 5F).

To investigate the relationship between utrophin expression and the degree of damage in the mdx skeletal muscles, we analyzed the expression levels of utrophin mRNA and the immunolocalization of utrophin (Fig. 5). In all muscles studied, the mean values of the expression levels for utrophin mRNA were higher in the mdx mice than in the B10 mice, and the 1.4- and 3.0-fold increases in the soleus and diaphragm muscles, respectively, were statistically significant \( (P < 0.05 \text{ and } P < 0.001) \) (Fig. 5A). In the mdx mice, the expression level of utrophin mRNA in the diaphragm muscle was \(-1.4\) to 2.7-fold \( (P < 0.05 \text{ and } P < 0.01) \) the levels in the other three muscles, and that in the gastrocnemius muscle was \(-2\)-fold \( (P < 0.01) \) the levels in the masseter and soleus muscles. The correlation coefficient between the degenerative areas of the skeletal muscles and the expression levels for utrophin mRNA in the mdx mice was 0.231, which was not significantly different from zero (Fig. 5B). In the B10 mice, immunostaining for utrophin was sporadically found in the periphery of myofibers (black arrowheads in Fig. 5, C and E). In the mdx masseter muscle, the periphery (black arrowheads) and the whole sarcoplasm of regenerative myofibers with central nuclei were immunostained for utrophin (Fig. 5D). The periphery of normal masseter myofibers without central nuclei (white arrowheads) was slightly stained, but the sarcolemma (arrows) was not stained (Fig. 5D). In the mdx diaphragm muscle, immunostaining for utrophin was observed in both the whole sarcoplasm and the periphery of normal and degenerative myofibers, and the immunostaining in the periphery (sarcolemma) of myofibers (black arrowheads) was more intense than that in the sarcolemma (Fig. 5F). The immunostaining patterns for utrophin in the gastrocnemius and soleus muscles were similar to those in the masseter muscle (data not shown).

**DISCUSSION**

In the course of skeletal muscle regeneration, quiescent satellite cells are activated to proliferate, and after several rounds of proliferation, the majority of satellite cells differentiate and fuse to form new myofibers or to repair damaged myofibers. We analyzed the expression of \textit{pax7} and m-cadherin as markers for satellite cells. On the basis of their

![Fig. 5](http://ajpcell.physiology.org/)
expression patterns and functions (4, 13, 15, 24), pax7 and m-cadherin are considered markers for quiescent, activating, and proliferating satellite cells. In the present study, the expression level of pax7 mRNA and the percentage of m-cadherin-positive cells to the total number of cell nuclei in the muscle tissues in all four muscles studied were greater in the mdx mice than in the B10 mice (Fig. 3B and 4E), suggesting that the activation and proliferation processes of muscle satellite cells occur more actively in mdx muscles than in B10 muscles. Reimann et al. (21) reported no significant difference in the percentage of m-cadherin-positive cells to the total number of cell nuclei in the soleus muscle between B10 and mdx mice, which appears to be inconsistent with our present data. This inconsistency is probably due to the difference in the age of mice in the present study (6 wk of age) from the ages of the mice that Reimann et al. (21) studied (~11–14.5 mo of age).

In the mdx mice, we found no significant correlation between muscle damage and expression level of pax7 (Fig. 3C) and no correlation between muscle damage and the ratio of satellite cells to the total number of cell nuclei in the muscle tissues (Fig. 4F), suggesting that the levels of activation and proliferation of muscle satellite cells are not correlated with the degree of damage in mdx skeletal muscles. However, because the correlation between muscle damage and the ratio of satellite cells to the total number of cell nuclei in the muscle tissues was nearly significant (when the t value for the correlation is >2.07, the correlation is statistically significant; the t value in the present study was 2.01), muscle satellite cells seem to be one of several factors influencing the degree of damage in mdx skeletal muscles, but not a great influencing factor. Gillis (12) proposed the following three factors that can lead to severe myofiber damage in the mdx diaphragm muscle: a large proportion of fast oxidative fibers having a large diameter, lifelong sustained activity, and forced lengthening during each contraction. Further studies are necessary to elucidate the factors that determine the degree of damage in mdx skeletal muscles other than the diaphragm muscle.

In both the B10 and mdx mice, the ratios of m-cadherin-positive cells to the total number of cell nuclei in the muscle tissues were greatest in the masseter muscles. This result indicates that the masseter muscle contains the largest pool of satellite cells, suggesting that the regeneration potential of the masseter muscle is much larger than that of the other muscles. If the same situation existed in the mdx muscles before the first episode of degeneration, it would not be surprising for the mdx masseter muscle to show much less damage than the mdx diaphragm muscle (Fig. 2E). In the present study, however, a clear and statistically significant negative correlation between muscle damage and the percentage of satellite cells could not be obtained (Fig. 4F). This result is probably due to the existence of other factors more influential than satellite cells. Masseter muscle reportedly has several unique characteristics (3, 5, 17, 26, 28, 32), and to these we may now add the characteristic of having a large pool of satellite cells and a large potential for regeneration.

Previous studies have reported that utrophin can functionally replace dystrophin and that the transgene expression of utrophin can prevent muscular dystrophy in mdx mice (2, 7, 30). Thus we had expected a high negative correlation between damage to the skeletal muscles and the expression level of utrophin mRNA in the mdx mice. Contrary to our expectation, the correlation coefficient between muscle damage and expression level was low and not significantly different from zero (Fig. 5B). In particular, the mdx diaphragm muscle exhibited the highest expression level for utrophin mRNA, although it was the most severely damaged by dystrophy. To determine whether upregulated utrophin cannot functionally replace dystrophin because it is not incorporated into the sarcolemma instead of dystrophin, we investigated the immunolocalization of utrophin (Fig. 5, C–F). Because intense immunostaining for utrophin was observed in the periphery of mdx myofibers (Fig. 5, D and F), we presumed that it was incorporated into the sarcolemma. In the present study, the expression level of utrophin mRNA in the mdx diaphragm muscle was ~1.4- to 2.7-fold the levels in the other three mdx muscles studied (Fig. 5A). To obtain complete disappearance of muscle damage, transgene expression is needed to reach ~11- and 25-fold the utrophin expression in mdx and normal mice, respectively (22). Thus it is most likely that the difference in the spontaneous upregulation of utrophin among different mdx muscles is too small to produce a difference in the degree of damage among different mdx muscles.

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REFERENCES
SATELLITE CELLS AND UTROPHIN IN MDX MUSCLE DAMAGE


