Analysis of Telomere Length in the Aged *mdx* Diaphragm with and without Transgenic IGF-I Expression

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Abstract

Chromosomal telomere length can help to indicate of the number of divisions a somatic cell has undergone. Telomere length was assessed in diaphragm muscles from *mdx* mice and *mdx* mice expressing high levels of insulin-like growth factor I (*mdx:mIgf*$^{+/+}$) to test if the hypertrophy and hyperplasia observed in the *mdx:mIgf*$^{+/+}$ diaphragm was associated with increased satellite cell division and a detectable change in telomere length. Southern blotting of digested genomic DNA was performed using a probe for telomeric repeats. DNA from young and aged *mdx* and aged *mdx:mIgf*$^{+/+}$ diaphragms displayed similar patterns of telomere repeat fragments. Therefore, no difference in number of satellite cell divisions could be detected. We postulate that inherently long telomere length and telomerase activity which are present in murine muscle obscure the telomere length measurement even when high levels of muscle regeneration exist. Therefore, the effect of IGF-I on cell replicative age will need to be determined in a species without telomerase, such as the canine model for muscular dystrophy.

Keywords: muscular dystrophy, *mdx* mouse, muscle regeneration, satellite cells, telomere length, insulin-like growth factor I.

Non-standard abbreviations: IGF-I, Insulin-like Growth factor I, *mdx:mIgf*$^{+/+}$, transgenic *mdx* mice expressing *mIgf-I, mIgf-I*, gene expressing IGF-I

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Introduction

Duchenne muscular dystrophy (DMD) is an X-linked disorder in which the loss of dystrophin causes muscle weakness and fragility and leads to premature death. Muscles from dystrophic patients undergo heightened cycles of damage and regeneration, and require continued replenishment of myonuclei from resident satellite cell pools [4]. Over time, muscle is replaced by fibrotic tissue, suggesting that the increased necessity for muscle repair associated with muscular dystrophy could eventually deplete the pool of satellite cells. In the *mdx* mouse, an animal model for DMD [25], the diaphragm most resembles muscles from human DMD patients in that only 50% of the muscle fibers remain by 14-16 months of age, and the balance is composed of fibrotic tissue [26]. Recent studies have shown that transgenic expression of insulin-like growth factor I (IGF-I) (*mdx:mIgf*$^{+/+}$ mouse) prevents fibrotic infiltration in the *mdx* diaphragm, where instead, there is dramatic hyperplasia of muscle fibers and increased overall muscle size at 14 months of age [2]. We have proposed that the increased fiber number and size is due to the promotion of muscle regeneration by IGF-I, which is critical to the proliferation and differentiation of satellite cells [14,15]. These results suggest that the satellite cell pool is not depleted in murine dystrophic tissue. However, it is not known if the increased muscle size can be maintained for the life of the animal (~2 years). This is a critical issue in the potential treatment of human patients with interventions to increase regenerative capacity. Even though the satellite cell population might not be depleted in the lifespan of a mouse, it is an open question whether or not increased regenerative capacity would result in premature senescence of muscle satellite cells within the human lifespan.

Telomere length proves to be an excellent indicator of the number of cell divisions [17,28]. A number of studies have shown that telomere length in human dystrophic muscle is significantly reduced compared to samples from healthy muscle [11,12]. In mice, however,

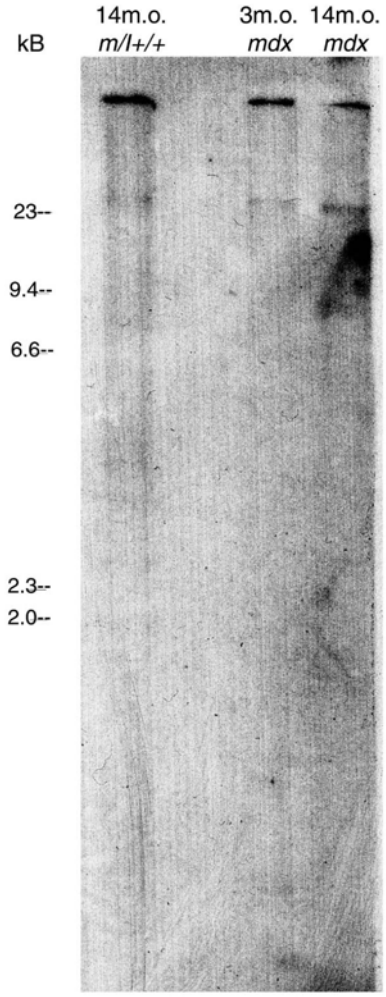

Figure 1. Telomere length analysis by southern blot of telomere repeat fragments. Genomic DNA from 14 month old mdx:mIgf$^{+/+}$ *diaphragms (*m/I+/+) *displayed no difference in telomere repeat fragments compared to genomic DNA isolated from 3 or 14 month old* mdx *diaphragms. In all cases, distinct bands were detected at ~23kB and at >23kB.*

the number of base pairs lost per division is minimized by telomerase activity which tends to maintain telomere length, even after several divisions, thereby obscuring the correlation between replicative age and telomere length [23,28].

In contrast to normal murine muscle, muscles from the *mdx* mouse exhibit increased repair processes, and heightened satellite cell activation and proliferation. The diaphragm from the *mdx:mIgf*$^{+/+}$ mouse provides an extreme case of increased regeneration and satellite cell division [2]. Therefore, we measured telomere length in 14 month old diaphragms from the *mdx* and *mdx:mIgf*$^{+/+}$ transgenic mice in order to test if the presence of IGF-I and the resultant increase in fiber number over time was associated with a decrease in telomere length.

Methods

DNA samples. All animal experiments were approved by the University of Pennsylvania's Institutinal Animal Care and Use Committee. Diaphragms were dissected from euthanized 3 month old *mdx* and 14 month old *mdx* and *mdx:mIgf*$^{+/+}$ mice and rapidly frozen in liquid nitrogen (n=3 for each condition). At this age, there was significant fibrosis in the *mdx* muscle, whereas the diaphragm from the *mdx:mIgf*$^{+/+}$ mice displayed little fibrotic infiltration [2]. Muscle DNA was isolated by proteinase K/SDS digestion and high salt extraction.

Southern blotting. The resultant genomic DNA was pooled for each condition and an aliquot (3 μg) was digested with the restriction enzymes Rsa I and Hinf I (New England Biolabs, Beverly, MA) which have short recognition sequences present in non-telomeric DNA. The digested DNA was separated by 0.7% agarose gel electrophoresis. The gel was subjected to southern blotting using a P32-labeled oligonucleotide probe specific for telomeric repeats $(TTAGGG)_4$. Determination of telomere length was achieved after the exposure of the blot to X-ray film, using the migration pattern of molecular weight marker on the original gel.

Results and Discussion

These experiments were used to determine if increased regenerative capacity by IGF-I expression would cause a decrease in telomere length of cells resident in the muscle. The Southern blot presented shows that the most prominent band (>23kB) remains in young and aged mdx diaphragm DNA, as well as in *mdx:mIgf*$^{+/+}$ diaphragm DNA (Figure 1). No other prominent bands were observed, and the intensity of the remaining smears of DNA in all samples was below the level of detection. Therefore, no significant difference in telomere length is apparent between DNA from young and aged *mdx* diaphragms. Further, in the increased regenerative capacity driven by IGF-I expression did not promote a detectable loss of telomeric DNA in the *mdx:mIgf*$^{+/+}$ diaphragms.

These results suggest that the satellite cell pool in dystrophic tissue is not depleted over time. Instead, it is possible that the signals which activate regeneration diminish over time. With sufficient stimulation by exogenous IGF-I, the satellite cell proliferation more than compensates for the need for fiber regeneration. However, the replicative capacity of muscle satellite cells is not unlimited. In normal muscle, it is sufficient to maintain muscle mass as long as existing muscle fibers can generate the appropriate signals for activating them [1, 5]. With aging, we and others have proposed that the limiting factor in muscle regeneration is the availability of growth and repair inducing growth factors, including IGF-I and hepatocyte growth factor [3,7,20,24]. Satellite cells extracted from aged muscle can still respond to these factors if they are provided [1,6]. However, it is unclear what the limiting factor is

in dystrophic muscle. Studies have shown that the mitotic potential of satellite cells from young muscular dystrophy patients is comparable to that found in human senescence, suggesting that the heightened necessity for muscle repair associated muscular dystrophy could eventually deplete the pool of satellite cells [11]. The results are clearly different in the murine model for muscular dystrophy, which shows no diminishment of telomere length after heightened satellite cell proliferation and differentiation driven by high levels of IGF-I expression [2].

Because satellite cells represent only a small proportion of the cell population in muscle (1-5%) [4], it is possible that any loss of telomere length in these cells would be masked by DNA from the post-mitotic myonuclei, which contain the majority of DNA in muscle. However, the repair processes of muscle rely on the proliferation of satellite cells, and so the "history" of proliferative events is contained within the surviving muscle nuclei. In a tissue which has undergone extreme degeneration and regeneration, such as the dystrophic diaphragm, this history should be indicative of the increased repair which has occurred during the life of the animal. Even if a difference could not be detected between the young and aged *mdx* diaphragms, the amplification of fiber number which has been seen in the *mdx:mIgf*$^{+/+}$ diaphragm could have perturbed the number of cell divisions experienced by the satellite cells to a point which diminished telomere length.

The presence of telomerase activity in the mouse could also obscure the measurement of telomere length [23]. Although the loss of telomeres still occurs, telomerase can resynthesize and repair the lost segments of DNA. However, it is not clear that telomerase activity is a significant protector against cell senescence or telomere length in normal mice. In studies which have measured telomerase activity in the mouse, there is little telomerase activity during periods without growth or regeneration [13]. Further, telomere length in the mouse is extremely long compared to other species (>23kB) [18]. In mice which are telomerase-deficient, several generations must occur prior to the appearance of severely shortened telomeres and significant impairments to cell division [16].

While we have shown that there is no difference in telomere length in the dystrophic mouse diaphragm, the issue still remains in the human dystrophies. First, the presence of telomerase, although debated in its relevance, is not present in human cells, and so telomere length remains a good indicator of human cell division [9,17]. Second, the lifespan of a mouse is less than 3% of the mean human lifespan, and so slow processes might not become apparent within the lifespan of the mouse. The fact that IGF-I expression in the mouse promotes increased functionality is also plagued by the same limitations. If IGF-I is improving the function of dystrophic muscle but simultaneously depleting the satellite cell pool, then such treatments of human patients would allow only transient improvement, followed by a precipitous decline in muscle function. This possibility is also true for other interventions which enhance satellite cell proliferation, such as myostatin inhibition. Recent reports have demonstrated that muscles from *mdx* mice treated with a systemic inhibitor of myostatin action [5] or crossed with a mouse lacking myostatin expression [27] have increased muscle mass and force generating capacity and decreased serum creatine kinase levels. These results are quite similar to those observed with IGF-I expression [2]. Thus, the issue of satellite cell depletion needs to be addressed in an animal model which is more like humans. The dog provides such a system, in which there is marked telomere shortening [21] over the lifetime of the animal. Second, fiber populations and the scale of the muscles are closer to humans. Third, the dystrophic canine model is very similar to human DMD in its severity and prognosis [8,10,19,22].

In conclusion, although telomere length could provide some measure of whether IGF-I is depleting a satellite pool by driving proliferation, this question cannot be pursued conclusively in the mouse. Prior to moving forward in combating muscular dystrophy with the utilization of interventions which increase regenerative capacity such as IGF-I, it is critical to address the true outcome in a more appropriate animal model.

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