1. Introduction

The muscular dystrophies form a group of disorders that are characterised by progressive weakness and wasting of the musculature due to continuous cycles of degeneration–regeneration of skeletal muscle. Duchenne muscular dystrophy (DMD) is a very severe disease appearing before 4 years of age and lethal by the end of the second decade [1,2]. This X-linked recessive disorder is caused by the mutation of a gene encoding the protein dystrophin [3]. This protein, which is absent in DMD patients, has been localised to the inner surface of the sarcolemma in skeletal muscle. It binds the cytoskeletal actin filament by its N-terminal, and a protein complex consisting of several subcomplexes (dystroglycan complex, the 25 kDa dystrophin-associated protein, sarcoglycan complex and dysferlin) by its C-terminus [4–6]. The extracellular domain of the dystroglycan complex binds to laminin-2, thereby linking the actin-based cytoskeleton via dystrophin to the extracellular matrix; see for a review [7].

Another group of muscular dystrophies is represented by the limb girdle muscular dystrophies (LGMDs) subdivided in two types: the autosomal dominant LGMDs (LGMD 1) and the autosomal recessive LGMDs (LGMD 2). Within the last decade, eight different forms of LGMD 2 have been mapped on different chromosomes. Some of the responsible genes have been cloned and the responsible protein defect identified [8–12]. LGMD 2C is a relatively severe childhood muscular dystrophy, which has clinical and pathological features similar to DMD [8,13]. Although the progression of the disease is inevitable, its severity is variable from one sibling to another and from one family to another. The disease is linked to chromosome 13q [14] and is caused by the mutation of a gene which encodes γ-sarcoglycan, a protein of the sarcoglycan complex [15].

In both LGMD 2C and DMD the primary defect is associated with a general or partial secondary deficiency of other dystrophin-associated proteins [16–19]. In DMD, it is currently thought that the absence of dystrophin results in a weakening of the cell membrane that can be more easily
Table 1
LGMD 2C and DMD patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>LGMD 2C</th>
<th>DMD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3116</td>
<td>2864</td>
</tr>
<tr>
<td></td>
<td>2872</td>
<td>2975</td>
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<tr>
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<td>3030</td>
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<tr>
<td></td>
<td>2736</td>
<td></td>
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<tr>
<td></td>
<td>2710</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>P1</td>
<td>P7</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>P9</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>P10</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>P11</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2</td>
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<tr>
<td>Clinical stage</td>
<td>II</td>
<td>IV</td>
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<tr>
<td></td>
<td>II</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>V</td>
</tr>
</tbody>
</table>

*LGMD 2C: limb girdle muscular dystrophy 2C DMD: Duchenne muscular dystrophy.
Clinical stage as defined by Gaidon-Macherand [30] and Walton [26], modified by Ben Hamida et al. [13].

In LGMD 2C, it is thought that the sarcoglycan complex assembly is dependent on the simultaneous synthesis of each sarcoglycan protein including the γ-sarcoglycan [21]. It is clear that dystrophin and the associated-protein complex are essential to maintain muscle integrity. The absence of any protein of this complex triggers a common process to muscular dystrophies, a continuous degeneration of the skeletal muscle. In response to this degeneration, satellite cells are activated and undergo several rounds of division before fusing to form new muscle fibres or to regenerate necrotic parts of pre-existing fibres [22]. As the disease progresses, the regenerative activity of the muscle declines, muscle fibres are gradually lost and are replaced by connective and adipose tissues.

In a previous study [23], by measuring the length of telomeric fragments in the myonuclei of muscle from healthy donors of different ages we have shown that normal skeletal muscle is a relatively stable tissue. The telomeres which are protein-DNA structures at the ends of eukaryotic chromosomes are implicated in chromosomal integrity and cellular ageing [24,25]. Each time a satellite cell divides, a small piece of the telomeric DNA is lost limiting the number of divisions these cells can make [26]. Therefore in skeletal muscle as in other tissues the measure of telomere length can be used to record the 'replicative history' of the tissue [23,27,28]. During the lifespan of DMD or LGMD 2C patients, the satellite cells will be forced to undergo repeated rounds of cell divisions to repair the continuous muscle damage and will be incorporated into muscle fibres. One of the mechanisms that might contribute to the reduced regenerative activity observed in the acute phase of the diseases is the premature senescence of satellite cells due to an increased muscle fibre turnover.

In this study, we have used an antibody specific for foetal myosin heavy chains to identify regenerated fibres in muscle of DMD and LGMD 2C patients [29]. We have also used the length of telomeric DNA to quantitate the intensity of muscle cell turnover in these dystrophic muscle tissues. Our results show that as soon as the first clinical symptoms of the disease become apparent the muscle has already undergone extensive regeneration as demonstrated by a reduction in minimal telomere length at a size similar to those found in normal muscle from old subjects. This reduction in telomere length will give an indication of the past history of the pathological muscle, i.e. the extent of regeneration this muscle has undergone. This is complementary to the histological and immunological data that will give an indication of the number of fibres which were undergoing regeneration when the biopsy was taken.

2. Material and methods

2.1. Muscle samples

Biopsies from 22 individuals were obtained during diagnostic or surgical procedures, in accordance with the French and Tunisian legislation on ethical rules. Healthy muscle biopsies were obtained during orthopedic surgery from the quadriceps of 12 patients who showed no signs of neuromuscular disorder. DMD and LGMD 2C patients were followed and diagnosed in the National Institute of Neurology at Tunis (Tunisia) (Table 1). The age of onset was defined by asking the parents to answer a questionnaire similar to that described by Dubowitz [30]. Muscle biopsies were obtained from DMD quadriceps and LGMD 2C deltoid muscles. The selected age range (4–13 years) of these patients coincides with the development and the clinical expression of DMD and LGMD 2C muscular dystrophies [2,13]. After sampling, all the biopsies were frozen in deep-cooled isopentane and stored at −80°C.

2.2. Immunohistochemistry

Sections (6 μm thick) were obtained from frozen samples and stained with haematoxylin and eosin or treated for immunocytochemistry. For immunocytochemistry, non-specific binding sites were blocked for 1 h at 37°C with non-immune serum. Sections were then incubated for 1 h at 37°C with monoclonal antibodies specific for foetal (WB-MHCn, Novocastra) myosin heavy chain used at dilution of 1/5. Specific antibody binding was revealed with peroxidase
### Table 2
Minimal TRF* lengths of muscle tissue from patients with muscular dystrophy and control donors

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age (years)</th>
<th>Minimal TRF length (kbp)</th>
<th>Sample</th>
<th>Age (years)</th>
<th>Minimal TRF length (kbp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>0.75</td>
<td>7.12</td>
<td>LGMD 3C</td>
<td>P1</td>
<td>5</td>
</tr>
<tr>
<td>C2</td>
<td>2.5</td>
<td>7.06</td>
<td>P2</td>
<td>8</td>
<td>5.99</td>
</tr>
<tr>
<td>C3</td>
<td>5</td>
<td>5.99</td>
<td>P3</td>
<td>10</td>
<td>4.55</td>
</tr>
<tr>
<td>C4</td>
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<td>7.27</td>
<td>P4</td>
<td>10</td>
<td>5.69</td>
</tr>
<tr>
<td>C5</td>
<td>15</td>
<td>6.63</td>
<td>P5</td>
<td>11</td>
<td>5.01</td>
</tr>
<tr>
<td>C6</td>
<td>20</td>
<td>7.42</td>
<td>P6</td>
<td>13</td>
<td>4.76</td>
</tr>
<tr>
<td>M1</td>
<td>9.2 ± 7.6</td>
<td>7.06 ± 0.68</td>
<td></td>
<td>9.5 ± 2.7</td>
<td>5.30 ± 0.05</td>
</tr>
<tr>
<td>C16</td>
<td>70</td>
<td>6.67</td>
<td>DMD</td>
<td>P7</td>
<td>4</td>
</tr>
<tr>
<td>C17</td>
<td>71</td>
<td>5.97</td>
<td>P9</td>
<td>8</td>
<td>5.85</td>
</tr>
<tr>
<td>C18</td>
<td>73</td>
<td>6.17</td>
<td>P10</td>
<td>9</td>
<td>4.65</td>
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<tr>
<td>C19</td>
<td>74</td>
<td>6.32</td>
<td>P11</td>
<td>13</td>
<td>4.42</td>
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<tr>
<td>C20</td>
<td>79</td>
<td>6.38</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C21</td>
<td>86</td>
<td>6.07</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>75.5 ± 6.0</td>
<td>6.25 ± 0.25</td>
<td></td>
<td>7.8 ± 3.5</td>
<td>5.31 ± 0.93</td>
</tr>
</tbody>
</table>

*TRF: telomeric restriction fragment. LGMD 3C: limb girdle muscular dystrophy 2C. DMD: Duchenne muscular dystrophy.

Averages are the mean ± SD.

*Significantly different from values of M1 group: a: P = 0.0038; b: P = 0.008.

*Significantly different from values of M2 group: c: P = 0.005; d: P = 0.04.

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**Fig. 1.** Histological changes and fetal myosin heavy chain reactivity in Duchenne muscular dystrophy. Serial cross-sections from the quadriceps muscle of a 4-year-old DMD patient P7 (A, C) and a 13-year-old DMD patient P11 (B, D) stained with haematoxylin-eosin (A, B) and treated with anti-fetal myosin heavy chain antibody (C, D). Magnification: ×500.
using the avidin-biotin technique (DAKO).

2.3. Isolation of genomic DNA

Muscle samples were ground to a powder in a liquid nitrogen-chilled mortar and suspended in protease K digestion buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA pH 8, 0.5% SDS) containing 0.1 mg proteinase K/ml. The lysates were incubated at 50°C overnight. Nucleic acid was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1 (vol./vol./vol.)). DNA was precipitated vol./vol. with ammonium acetate 7.5 M/ethanol 100% (0.5:2 (vol./vol.)), then with ethanol 70% and finally with ethanol 100%. DNA was dissolved in TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5) and stored at 4°C.

2.4. Analysis of telomere length

DNA samples obtained from muscle tissue were digested with the restriction enzyme HinfI for 2 h at 37°C. This enzymatic digestion generates terminal restriction fragments...
2.5. Statistical analysis

Statistical analysis and linear regression were computed using GraphPad Prism (v. 2.01). Statistical significance was assessed by Student’s t-test. P < 0.05 was taken as significant. Data were expressed as means ± SD.

3. Results

3.1. Morphological and immunohistochemical analyses of human muscle tissues from DMD and LGMD 2C patients

In this study, the biopsies were obtained from the quadriceps of DMD patients and from the deltoid muscles of LGMD 2C patients since these are the muscles first affected during the progression of the disease [13,30]. All biopsies showed characteristic pathological anomalies.

For DMD patients, the histological changes increased significantly with the age of the subject and the progression of the disease (Fig. 1). In Fig. 1A, it can be seen that the majority of the muscle fibres were polygonal and regular in size. The amount of connective tissue was low and there was no evidence of adipose tissue. Small foci of actively regenerating fibres could be observed. In older patients, the pathological changes in the muscle biopsies were much more significant. As shown in Fig. 1B, in the biopsy of a 13-year-old patient P11, the fibres were more rounded, there was a larger dispersion of their diameters and an infiltration of both connective tissue and fat. The small regenerating fibres tended to be isolated and were fewer in number than that observed for the 4 year old DMD boy (Fig. 1A).

For LGMD2C patients (Fig. 2), the histological pattern varied sometimes between patients of the same age (10-year-old LGMD 2C patients P3 and P4). Although these two subjects were the same age, the histological pattern of the two deltoid biopsies was very different. Patient P4 had a relatively normal looking muscle with only a few pathological changes consisting of a small number of regenerating fibres and a slight variability in fibre diameter. These muscle fibres had a polygonal shape and were tightly packed together. The muscle biopsy of the patient P3, although of the same age, was much more severely affected with a marked variation in fibre diameter, the presence of round dark-staining fibres, development of endomysial fibrosis, areas of necrotic and regenerating fibres. Muscle biopsies of patients P1 and P2 had similar histological lesions as the biopsy of patient P7, whereas those of P5 and P6 had a more advanced profile with fewer foci of regenerating fibres (data not shown).

The foetal isoform of MHC (fMHC) which is not normally expressed beyond the first month of post-natal life [52], was detected in the regenerated muscle fibres in muscle biopsies of both DMD (Fig. 1C,D) and LGMD 2C patients. The percentage of regenerating fibres expressing fMHC varied from 38–47% in the DMD biopsies and 37–
48% in LGMD 2C biopsies. These values were not related either to the stage of the disease or to the age of the subject. This would suggest that there is a continuous process of regeneration involving over a third of the muscle fibres even in the youngest subjects.

3.2. Marked decrease of telomere lengths in DMD and LGMD 2C skeletal muscle

In order to determine the rate of telomere loss in the skeletal muscle of dystrophic subjects, we have analysed muscle biopsies obtained from DMD and LGMD 2C patients and control subjects of different ages (Table 2). A representative autoradiograph is shown in Fig. 3. The minimal TRF lengths of the LGMD 2C and DMD groups presented similar ranges of values and were significantly shorter than those of both control groups M1: young donors (P = 0.0008 and P = 0.008, respectively) and M2: old donors (P = 0.005 and P = 0.04, respectively).

When the minimal telomere length of dystrophic muscle tissue was analysed as a function of donor age (Fig. 4), a significant correlation was observed (m = −187 ± 49 bp/year, r = 0.64, P = 0.005) (the slope (m), correlation coefficient (r), and P value tested the null hypothesis that the slopes are 0). However, while in these two dystrophic groups the minimal telomere length tended to decrease with the age of the patients, we also notice that some patients with similar ages could present very different values of telomere length. For example in the LGMD 2C group, the two children P3 and P4 both aged 10 years had minimal telomere lengths of 4.55 and 5.69 kbp respectively (Table 2). Similarly in the DMD group, the children P9 and P10 aged 8 and 9 years had minimal telomere lengths of 5.85 and 4.65, respectively.

4. Discussion

A decrease in telomere length has been reported to occur during the in vivo ageing of various normal human tissues such as skin, intestinal mucosa, peripheral lymphocytes and skeletal muscle [23,27,28,33]. Although the mean value of telomere length has been shown to be a good marker of replicative capacity for mitotic tissues, it has not been proved to be a pertinent value to measure the small loss of telomeric DNA in tissue such as skeletal muscle which is considered to be post-mitotic [23]. In a previous study we have shown that the minimal telomere length is a more sensitive value to determine the low rate of telomere shortening which occurs in normal muscle tissue as a result of nuclear turnover and/or regeneration [23]. The measure of the minimal TRF length allows one to remove from the telomeric signal the longest TRFs corresponding to the telomeres of the post-mitotic myonuclei which had been included in muscle fibres early during development and had consequently undergone very few mitosis before differentiation as compared to nuclei of satellite cells incorporated at a later date. Satellite cells which represent approximately 5–6% of the total muscle nuclei in the adult are the only muscle cells capable of dividing [34]. During normal in vivo ageing, the low rate of telomere shortening of 13 bp/year confirms a small turnover of muscle nuclei [23].

Telomere length has also been used in several studies to demonstrate an accelerated cellular turnover in diseased cells and tissues of subjects affected by HIV, chronic hepatitis or liver cirrhosis [35,36]. In DMD and LGMD 2C the onset of muscle weakness and wasting is often preceded by a phase of muscle hypertrophy, particularly of the calf muscles [30] and some cases even show a universal prominence of their muscles. This increase tends to occur when the children are still ambulant and is less prominent once ambulation is lost. A similar hypertrophy has been recently described for one of the mouse LGMD models (pers. commun.).

In this study, we have evaluated the rate of loss of telomeric DNA in dystrophic skeletal muscle and found that it is 14 times greater than the rate of telomere loss measured in muscles of healthy subjects (187 bp/year versus 13 bp/year) [23]. This accelerated rate of telomere loss in DMD and LGMD 2C muscles can be explained by the active process of regeneration involving more than a third (37–48%) of the muscle fibres observed at all stages of the disease except in terminal stages when regeneration has become abortive. In association with the fMHC expression, the shorter telomere length values confirm the presence of an active process of satellite cell division and regeneration of new fibres in the dystrophic muscles. Previous studies have shown that the proportion of regenerating fibres in DMD muscles are independent of the stage of the disease [29,37]. Although the fMHC isoform is a marker of muscle regeneration, it does not permit one to evaluate the number of times the muscle
has regenerated and thus the progressive evolution of the disease. Since telomeres shorten at each cell division, the measurement of telomere length is a good indicative marker to estimate the number of cycles of degeneration–regeneration that the muscle has undergone.

All dystrophic muscles we examined showed a significant reduction in telomere length compared to that in control groups. The average minimal TRF lengths were respectively 7.06 ± 0.68 and 6.23 ± 0.25 kbp in young and older healthy individuals and 5.30 ± 0.60 and 5.31 ± 0.93 kbp in LGMD 2C and DMD individuals.

Although the muscles of the youngest dystrophic patients (P1, P7) or of patients P2 and P4 had a quasi-normal appearance, their minimal telomere lengths were shorter than those of young healthy subjects and were in fact very similar to the minimal telomere length values measured in muscles of the oldest control subjects. In the muscle of the youngest dystrophic patients (P1, P2, P4 and P7) the minimal TRF lengths had already decreased by approximately 1100 bp. This is even more than the amount of telomeric DNA that had been lost in all of our subjects who were older than 60 years, indicating that in these young patients the muscles have already undergone a significant number of cycles of regeneration. Moreover for patients P1 and P2, this extensive turnover of muscle nuclei had already occurred prior to the appearance of the clinical symptoms since the muscle biopsies had been made at the onset of the first clinical manifestations and showed very few signs of morphological alterations. It is interesting to note that the severity of these diseases is not always correlated with the age of the child but there is a strong correlation between the clinical severity, morphological changes and decreased telomere length. This is very well demonstrated by the two subjects P3 and P4 who were both 10 years old, but clinical symptoms were much more pronounced in P3 than in P4 and the telomeres were much shorter in P3 than P4, demonstrating that the muscles of P3 had undergone many more cycles of regeneration than P4. This also reinforces the assumption that the decrease in TRF length gives an accurate reflection of the past regenerative history of the muscle.

As the disease progresses the skeletal muscle will be subjected to continued cycles of degeneration and regeneration of the muscle fibres. This progression is accompanied by an increase in the pathological lesions observed in the muscle (rounded fibres with variable diameters, fibrosis and infiltration of adipose tissue). During these cycles of regeneration the satellite cells proliferate, resulting in a progressive shortening of the telomeric DNA in these fibres. This was confirmed in our study by a further loss of 1200 bp measured in the oldest patients in our study (P5, P6, P11). It should be noted that at this stage of the disease, no foci of active regeneration were observed. This would suggest that the regeneration has become abortive due to a progressive senescence of the satellite cells. These cells are no longer able to divide and to participate in ongoing regeneration. The fact that very few satellite cells with a greatly reduced life span could be isolated from these biopsies would support this hypothesis [38] (unpublished data from our group).

In conclusion, our results provide experimental data demonstrating that muscle cells have been subjected to intense proliferation and regeneration in dystrophic patients even before the appearance of the first clinical symptoms. While the degeneration of muscle fibres is the consequence of a primary genetic defect, the ultimate failure of regenerative activity is due in part to the normal response of the satellite cells to a massive proliferation inducing a reduction in the length of the muscle telomeric DNA and a premature senescence of the satellite cells. However, in this complex system it is evident that other factors still to be determined are involved in the decline in regenerative capacity which characterise these diseases.

Acknowledgements

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