Telomerase Activity in Regenerated Normal Muscles: Effects of Repeated Injuries

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Abstract

Duchenne muscular dystrophy (DMD) is a chronic, X-linked recessive disorder which causes progressive muscle wasting and weakness leading to an early death. Three progressive characteristics accompany the atrophy and coincident clinical weakness: active proliferation of endomysial connective tissue (fibrosis), muscle fiber atrophy and failure of myofiber regeneration. A factor that adversely affects new fiber formation is the reduced replicative capacity of satellite cells.

As the first step to verify if reduced telomerase activity and consequent chromosomal telomeric shortening could affect the proliferative capacity of satellite cells in mice, we measured telomerase activity in neonatal and adult muscle of normal mice. We detected telomerase activity by Telomerase PCR ELISA. Moreover we analysed telomerase activity in muscles which underwent repeated cycles of injury/regeneration by local infiltration of myotoxic agent (bupivacaine). The treatment was repeated up to three times and the muscles were analysed 3 days after the last injection.

In neonatal normal muscle the telomerase activity is elevated while in adult normal muscle the activity is absent. In three days regenerating muscle telomerase activity is measurable but much lower than in neonatal muscle. After 2 and 3 bupivacaine treatments the level of telomerase activity is comparable with that of adult normal skeletal muscle, even in muscle with high levels of proliferating myoblasts. These results suggest that repeated cycles of regeneration cause decrease of telomerase activity and consequently, chromosomal telomeric shortening in proliferating myoblasts.

Key words: Duchenne muscular dystrophy, regeneration, repeated muscle damage, telomerase.

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Duchenne muscular dystrophy (DMD) is a common, X chromosome-linked muscle disorder which causes progressive muscle wasting and weakness leading to continuous cycles of degeneration- regeneration of skeletal muscle. In response to this degeneration, activated satellite cells undergo several rounds of division and fuse to form new muscle fibres. The progression of the disease is due to the decline of regenerative activity of muscle so muscle fibres are gradually lost and are replaced by connective and adipose tissue. One of the mechanisms that might contribute to the reduced regenerative activity is the premature senescence of satellite cells [20], though this hypothesis is debated [15, 16]. Proliferative capacity of satellite cells can be analysed using telomere length which decreases during each round of cell division [5, 6] Telomeres are specialized structures at the end of eukaryotic chromosomes, vertebrate telomeres are composed of proteins and a hexameric sequence (TTAGGG) repeated for many kilobases [13], this structures are implicated in chromosomal integrity and cellular ageing [9]. Telomeres continuously shorten with successive cell divisions due to the inability of normal DNA polymerases to replicate the end of eukaryotic chromosome [1, 10]. Normal human cells undergo a finite number of cell divisions and ultimately enter a non dividing state called replicative senescence [12]. Shortening of telomeres has been proposed as a mitotic clock that regulates proliferative potential of somatic cells and the limited proliferative capacity of DMD myoblasts limits their ability to be genetically modified and used for myoblasts transplantation [7, 19].

An enzyme, called telomerase, provides the necessary enzymatic activity to restore and maintain telomere length [2]. Telomerase is a ribonucleoprotein which extends the 3' end of telomeres by *de novo* synthesis of the G-rich telomeric repeat DNA using a segment of RNA within its molecule as a template[8]. Telomerase is active

in germ line cells and stem cells but is repressed upon tissue differentiation during development [21]. It is not known if expression of this enzyme is similar or not in myoblasts derived from developing muscle and in satellite cell-derived myoblasts, which regenerate myofibers after adult muscle damage [17]. To this purpose, we measured telomerase activity in new born and adult muscles of normal mice, and in muscles which underwent repeated cycles of injury/regeneration by local infiltration of a myotoxic agent (bupivacaine).

Materials and Methods

We detected telomerase activity by Telomerase PCR ELISA (Roche). This method is designed for the highly sensitive detection of telomerase activity in cell extracts from cell cultures and other biological samples. The Telomerase PCR ELISA, developed by ROCHE, is an extension of the original method described by Kim et al., it allows highly specific amplification of telomerase-mediated elongation products combined with non radioactive detection following a ELISA protocol.

Preparation of extracts from tissues

Normal and regenerating muscles of adult mice and normal muscles of new-born mice were frozen in liquid nitrogen and stored at -80°. Cryostat sections of 10 µm were prepared and transferred into a sterile reaction tube with 200 µl of lysis reagent, than, after incubation on ice and centrifugation at 16000 × g for 20 min. at 4°C, the supernatant was removed and transferred to a fresh tube. Regenerating muscles were obtained after intramuscular injection with 0.5% Bupivacaine solution.

TRAP assay (Telomeric Repeat Amplification Protocol)

An aliquot of tissue extract corresponding to 10 µg of total proteins was mixed with the Reaction mixture of the kit to perform the TRAP assay. In the first step telomerase, if present in the tissue extracts, adds telomeric repeats (TTAGGG) to the 3' end of the biotin-labeled synthetic P1-TS primer. In a second step these elongation products are amplified by PCR using the primer P1-TS and a second primer P2, generating PCR products with the telomerase-specific 6 nucleotide increments.

The biotin-labeled P1-TS primer allows easy analysis of the amplification products, obtained after elongation and amplification, by Southern hybridization to have a proof for the presence of the typical 6-nucleotide-ladder.

An aliquot of the PCR product was mixed with loading dye containing bromophenol blue and xylene cyanol and separated by polyacrylamide gel electrophoresis using a 12% non denaturing acrylamide gel. The biotinylated PCR products were than blotted onto a positively-charged nylon membrane and visualised by chemiluminescence using a non isotopic detection kit (BrightStar BioDetect AMBION).

ELISA procedure

An aliquot of PCR product was denaturated and hybridized to a digoxigenin-(DIG)-labeled,telomeric repeat-specific detection probe. The resulting product was immobilized via the biotin labeled primer to a streptavidin-coated microtiter plate. The immobilized PCR product was detected with an antibody against digoxigenin (anti-DIG-POD) that was conjugated to peroxidase. Finally, the probe was visualized by virtue of peroxidase metabolizing TMB (3,3',5,5'-tetramethyl benzidine) to form a colored reaction product. The absorbance values related to telomerase activity were reported as the absorbance measured at a wave-length of 450 nm reading against blank (reference wave-length of 690 nm). According to the manufacturer, samples are regarded as telomerase-positive if the difference in absorbance ($A_{450\ nm}$-A_{690nm}) is higher than 0.2.

Table 1. Telomerase activity of developing and adult skeletal muscles of mice.

	N°	Absorbance ± S.D.
New born	3	0.59 ± 0.08
2 day-born	4	0.70 ± 0.05
Adult	4	0.03 ± 0.01

Results and Discussion

In a first series of analysis we detected telomerase activity in normal muscles of new born, 2-days born and adult mice to check the sensitivity of TRAP assay in muscle tissue.

In skeletal muscles of new born mice the telomerase activity is well elevated over the backgrounds of the blanck reactions, while, as expected, in adult muscles the activity is absent (Table 1). The presence of telomerase activity in neonatal myoblasts was confirmed by analysis of telomerase activity in muscle cultures from new-born mice (data not shown). These results are expected because telomerase is active during embryogenesis and is downregulated during development [14]. Furthermore we measured telomerase activity in regenerating adult normal muscles to verify if reactivation of satellite-cells causes an increment of telomerase activity. To reactivate satellite cells we injected Tibialis anterior muscles of adult normal mice with bupivacaine, in fact it is well known that after myo-

Table 2. *Telomerase activity of early regenerating muscles after 1-, 2- and 3-cycles of damage-regeneration in adult mice.*

	N°	Absorbance ± S.D.
Adult	4	0.03 ± 0.01
1-cycle	4	0.23 ± 0.09
2-cycles	5	0.12 ± 0.04
3-cycles	4	0.12 ± 0.03

toxic damage the muscle fibers degenerate and there is an inflammatory reaction which is accompanied by satellite cells activation [3, 4]. The treatment was repeated one, two times and the muscles were analysed after 3 days from the last injection. In the last case we also detected the presence of proliferating myoblasts using the polyclonal antibody against MyoD, a trascription factor expressed in proliferating myoblasts and myotubes.

Table 2 shows that after a single marcaine injection in three-days regenerating muscles telomerase activity is measurable but the absorbance values are only slightly higher than the value suggested by the manufacturer as telomerase positive. On the other hand, after 2 and 3 bupivacaine treatments the level of telomerase activity decreases and is comparable with that of adult normal skeletal muscle. To verify if the absence of telomerase activity could be due to the absence of proliferating myoblasts we used anti-MyoD polyclonal antibody and we revealed several positive cells even in muscles, which are negative for telomerase activity. All togheter these results suggest that in myoblasts derived from satellite cells telomerase is dowregulated or permanently inhibited. On the other hand, it remains to be determined if the number of myoblast in our sample are or not below the level of detection of the sensitive Telomerase PCR ELISA test we used.

In conclusion, present result suggest that during regeneration satellite cells proliferate but do notexpress telomerase activity and so repeated cycles of regeneration could induce a progressive shortening of telomeric DNA and a premature senescence of satellite cells [18, 19].

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