Telomere shortening is associated to TRF1 and PARP1 overexpression in Duchenne muscular dystrophy

M’Hammed Aguennouza,1, Gian Luca Vitaa,1, Sonia Messinaa, Annamaria Camaa, Natalia Lanzanoa, Annamaria Ciranniа, Carmelo Rodolicoа, Rosa Maria Di Giorgiob, Giuseppe Vitaa,*

a Department of Neurosciences, Psychiatry and Anaesthesiology, University of Messina, AOU Policlinico, Messina 98125, Italy
b Department of Biochemical, Physiological and Nutritional Sciences, University of Messina, AOU Policlinico, Messina 98125, Italy

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Abstract

Telomere shortening is thought to contribute to premature senescence of satellite cells in Duchenne muscular dystrophy (DMD) muscle. Telomeric repeat binding-factor-1 (TRF1) and poly (ADP-ribose) polymerase-1 (PARP1) are proteins known to modulate telomerase reverse transcriptase (TERT) activity, which controls telomere elongation. Here we show that an age-dependent telomere shortening occurs in DMD muscles and is associated to overexpression of mRNA and protein levels of TRF1 and PARP1. TERT expression and activity are detectable in normal control muscles and they slightly increase in DMD. This is the first demonstration of TRF1 and PARP1 overexpression in DMD muscles. They can be directly involved in replicative senescence of satellite cells and/or in the pathogenetic cascade through a cross-talk with oxidative stress and inflammatory response. Modulation of these events by TRF1 or PARP1 inhibition might represent a novel strategy for treatment of DMD and other muscular dystrophies.

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1. Introduction

Telomeres are short, repeated sequences of TTAGGG located at the end of chromosomes which are involved in several essential biological functions (Cong et al., 2002). Telomeres contribute to the functional organization of chromosomes, participate in the regulation of gene expression, and serve as a molecular clock that controls the replicative capacity of the cells. In somatic cells, telomeres shorten with successive cell divisions, and it results in progressive genomic instability and altered gene expression, contributing to replicative senescence, apoptosis or neoplastic transformation (Blasco, 2005). Despite this apparently inevitable phenomenon, telomere length does not change considerably from generation to generation in mammals, indicating the existence of some mechanism to maintain telomeres. That mechanism turns out to be the expression of telomerase, a ribonucleoprotein enzyme complex composed of an RNA subunit and a catalytic protein subunit called telomerase reverse transcriptase (TERT), which is the rate-limiting factor for the enzyme activity (Shay and Wright, 2004). Telomerase synthesizes telomeric repeats onto chromosome ends by use of an endogenous ribonucleic acid as a template and provides the molecular basis for unlimited proliferative potential in the germline and also in somatic cells (Flores et al., 2006). The existence of a telomerase-independent mechanism, at least in cancer cells, further complicates this issue (Reddel, 2003). The shortening of telomeres may be due not only to loss of tandem repeats during replication, but also to direct oxidative damage to telomeres (von Zglinicki, 2002). Based on the evidence that oxidative stress may be a key mediator of the aging process in general, this suggests that telomere length...
could serve as a surrogate marker for the overall aging of cells and tissues, providing an index of biological age rather than just replicative history. Recently, some reports examining telomere length in peripheral blood mononuclear cells, as easily obtainable cells, have documented a correlation between shortened telomeres and some age-related diseases, such as heart failure (Oh et al., 2003), myocardial infarction (Brouilette et al., 2003) and atherosclerosis (Benetos et al., 2004), and poorer clinical outcome after stroke (Martin-Ruiz et al., 2006) and in Alzheimer’s disease (Honig et al., 2006).

Telomere-specific DNA-binding proteins have been put forward as additional candidates for the role of molecules modifying telomerase activity, and they have been suggested to play key roles in the maintenance of telomere function in mammals. The multiprotein complex known as shelterin or telosome includes two subunits, telomeric repeat binding factor-1 (TRF1) and TRF2, and also RAP1, Tin2, POT1 and TPP1, directly or indirectly associated with TRF1 or TRF2 and strongly pointing to a functional connection among these six telomeric proteins. The current data highlight that shelterin is not a static structural component, but a protein complex with DNA remodelling activity that acts together with several associated DNA repair factors. Telomere elongation by telomerase is repressed in cis by the TRF1 complex and overexpression of TRF1 in a tumour-cell line results in a gradual and progressive telomere shortening (de Lange, 2005). Conversely, the expression of a dominant negative allele of TRF1, which removes endogenous TRF1 from telomeres, leads to telomere elongation (van Steensel and de Lange, 1997).

It is assumed that the so-called t-loop of telomeric DNA has to become unfolded before telomerase can access the telomere and telomere elongation can take place. t-loop unfolding is achieved by inhibiting TRF1 and/or TRF2 binding. At least four members of the poly(ADP-ribose) polymerase (PARP) family, namely tankyrase (TANK)1, TANK2, PARP1, and PARP2, have been found in association with telomeric DNA and are able to poly(ADP-ribose)ate TRF1 and TRF2, thus blocking their DNA-binding activity (Cook et al., 2002; Dantzer et al., 2004; Muramatsu et al., 2007). PARP1 is the most studied enzyme involved in a number of pathways including DNA replication and repair, recombination, gene transcription, cell proliferation and death (De Boeck et al., 2009).

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease leading to loss of ambulation by the 13th year and to death, usually in early adulthood. The disease results from absence of the protein dystrophin, which is an essential component of the dystrophin–glycoprotein complex that maintains membrane integrity of muscle fibers by linking cytoskeleton to extracellular matrix (Dubowitz and Sewry, 2007). Although the primary genetic defect is known, how this mutation gives rise to the final disease status is not fully understood. Pathological hallmarks of the dystrophic process are in muscle necrosis, phagocytosis, infiltration of inflammatory cells and initial efficient regeneration with several rounds of division of satellite cells. As the disease progresses, the regenerative activity of the muscle declines, muscle fibers are gradually lost and are replaced by connective and adipose tissues. One of the mechanisms that might contribute to such reduced regenerative activity is the premature senescence of satellite cells due to an increased muscle fiber turnover (Webster and Blau, 1990). Despite its post-mitotic status, skeletal muscle tissue is not stable since satellite cells are constantly recruited following muscle damage. Satellite cells are muscle stem cells and are supposed to be the only proliferative cells in human skeletal muscle after birth, so that they are the only responsible for the addition of nuclei during in vivo muscle repair (Kadi and Ponsot, 2009). It has been hypothesized that telomere shortening in DMD muscle underlies satellite cell senescence, but investigations led to controversial results. Similar mean telomere lengths in normal and dystrophic muscles cast doubt on replicative aging as a cause of disease progression, although a mild age-dependent shortening of telomeres was found in DMD patients (Oexle et al., 1997). Conversely, a significant reduction in the minimal telomere length, a more sensitive value since it better reflects the extensive regeneration occurred by satellite cells activation (Decary et al., 1997), was found in DMD and limb girdle muscle dystrophy type 2C patients with a negative correlation with age (Decary et al., 2000). Recently, shortened mean telomere length has been reported in diaphragm and to a lesser extent in tibialis anterior muscles of aged mdx mice, the murine model of DMD, supporting a correlation to a more continuous muscle working and more severe histopathology (Lund et al., 2007). These findings reinforce the assumption that telomere shortening could potentially account for the declining regenerative ability of DMD muscle as the disease progresses. Very recently, telomere shortening has been detected in sporadic inclusion-body myositis, associated to a low myoblast proliferative rate and clonogenicity, providing new clues for future therapeutic strategies (Morosetti et al., 2008).

The possibility of limiting or preventing the replicative senescence of satellite cells represents a powerful therapeutic strategy for a number of skeletal muscle defects. Zhu et al. (2007) have recently described the production and characterization of an immortal human myoblast cell line, that has overcome replicative aging due to the expression of telomerase and cyclin-dependent kinase 4. The goal of the present work was to test the hypothesis that shorter telomeres, contributing to satellite cells senescence in DMD muscle, are associated to an altered balance of some telomeric molecules which control telomere length and telomerase activity, and modulate replicative status.

2. Materials and methods

2.1. Muscle biopsies

We studied vastus lateralis muscle samples, stored at −80 °C, from 20 patients with DMD (age range, 6 months to
9 years). DMD was diagnosed on clinical features, muscle biopsy with dystrophin analysis by immunocytochemistry and Western blot, and study of the dystrophin gene. The patients were divided into two subgroups, according to clinical severity: 9 asymptomatic patients ≤2.5 years of age (mean ± SD, 15.3 ± 7.9 months) and 11 symptomatic patients with progressively more severe histopathology ≥4 years of age (mean ± SD, 81.8 ± 18.6 months). Five muscle samples taken from healthy subjects (2–9 years of age), without muscle disorder and undergoing orthopedic surgery, were tested as controls. Their parents had given informed consent for the scientific use of the muscle biopsy. The Medical School Ethical Committee, University of Messina, authorized the study.

2.2. Telomere length measurements

Genomic DNA was extracted from muscle biopsy specimens performing a standard method. Mean and minimum telomere length were measured using the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostics, Milan, Italy). Briefly, 5 μg of genomic DNA was digested with a HinII/RsaI mixture (5 units/μg DNA) and separated on 0.8% agarose gels. Fractionated DNA fragments were transferred onto a nylon membrane (Roche Diagnostics) and hybridized with a digoxigenin-labeled telomeric probe, (TTAGGG)n. The hybridized probe was incubated with a digoxigenin-specific antibody covalently coupled to alkaline phosphatase. The immobilized telomere probe was visualized by a highly sensitive chemiluminescent substrate for alkaline phosphatase. The intensity of the hybridization was evaluated by densitometric analysis with Quantity One software (Bio-Rad Laboratories, Hercules, CA) and mean telomeric restriction fragment (TRF) length of a sample was estimated according to the formula \( \Sigma(OD_i)/(\Omega(OD_{Li}/L_i)) \), where \( OD_i \) is the intensity of hybridization signal, \( L_i \) is the length of the TRF in kilobase (kb) at the gel point \( i \) and \( L_t \) represents the mean molecular weight at the mid-point of each of 30 equal boxes of the telomeric smears in the range of 2–23 kb (Harley et al., 1990). The minimum telomere length was calculated using the Scion Image v4.03 software (Scion Corporation, Frederick, MD), as recently described (Ponsot et al., 2008). Mean telomere length comprises TRFs of the majority of postmitotic myonuclei, which had been incorporated in muscle fibers from birth and, consequently, have undergone only a few mitotic divisions before differentiation; minimum TRF length comprises TRFs from satellite cells and also from the myonuclei newly incorporated during the last mitotic cycles.

2.3. Immunoblotting analysis

Muscle samples were homogenized in lysis buffer (1 M Tris–HCl, pH 7.4, 15% Sodium dodecyl sulphate (SDS) and 5% β-mercaptoethanol) using a potter device on ice. The extracts were boiled for 5 min at 95 °C and centrifuged for 10 min at 14,000 rpm. Total proteins were determined with Lowry method. Protein samples (220 μg) were denatured in reducing buffer (62 mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromphenol blue) and separated by electrophoresis on 12% SDS polyacrylamide gel with kaleidoscope prestained standard proteins (Bio-Rad, Milan, Italy) to achieve a more accurate molecular weight determination. The separated proteins were transferred onto a nitrocellulose membrane using the transfer buffer (39 mM glycine, 48 mm Tris pH 8.3, 20% methanol) at 200 mA for 1 h. The membranes were stained with Ponceau S (0.005% in 1% acetic acid) to confirm equal amounts of protein and were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) and 0.1% Tween 20 for 1 h at room temperature, washed three times for 10 min each in TBS-0.1% Tween 20, and incubated with goat polyclonal antibody raised against a peptide mapping at the C-terminus of human TRF1 and with mouse monoclonal antibody against amino acids 764–1014 mapping at the C-terminus of human PARP1 (respectively, code sc-1977 and sc-8007; Santa Cruz Biotechnology, Santa Cruz, CA) respectively at a concentration of 1:1500 and 1:400 in TBS-0.1% Tween 20 overnight at 4 °C. After washing three times for 10 min each in TBS-0.1% Tween 20, membranes were incubated with peroxidase-conjugated anti-mouse or anti-goat antibody (Dako, Milan, Italy) at a concentration of 1:1500 and 1:1000, respectively, for 2 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer’s protocol (Amersham, UK). The protein signals were quantified by scanning densitometry using a bioimage analysis system (Kodak, Milan, Italy). The results were expressed as relative integrated intensity compared with control muscle measured with the same batch. Equal loading of protein was assessed by immunodetection of β-actin with a rabbit monoclonal antibody (Cell Signaling Technology, Beverly, MA) diluted 1:500 and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, Milan, Italy) diluted 1:15,000.

2.4. RNA isolation and real-time PCR

Total RNA was extracted from each muscle biopsy specimen using TRIzol reagent, followed by DNase I treatment (Invitrogen, Carlsbad, CA). The RNA quality and quantity were checked respectively on agarose gel and by spectrophotometry. 3 μg of total RNA from each sample was reverse-transcribed by Archive kit (Applied Biosystems, Milan, Italy). Generated cDNA was used as template for real-time quantitative PCR analysis using gene expression products according to the manufacturer’s recommendations. For each real-time PCR reaction, we used 2.5 μl of cDNA in a total volume of 50 μl. We performed reactions with a 7300 Sequence Detection System apparatus (Applied Biosystems)
to quantitatively compare the mRNA levels. TRF1, PARP1, human TERT (hTERT) and β-actin (as an endogenous control) assays were obtained from Applied Biosystems. Realtime PCR was performed in duplicate with 2× TaqMan Universal PCR Master Mix. The thermal cycling conditions consisted of one cycle each for 2 min at 50 °C and 10 min at 95 °C, and 40 cycles for 15 s at 95 °C and 1 min at 60 °C. All gene expression levels were normalized to β-actin mRNA level, which was determined simultaneously in the same tube. The comparative cycle threshold (Ct) method (Applied Biosystems) was used to analyse the data by generating relative values of the amount of target cDNA. Relative quantification (RQ) for these genes was expressed as fold variation over control, and was calculated by the ΔΔCt method, using control samples as calibrators.

2.5. Measurement of telomerase activity

Telomerase activity was measured by a telomere repeat amplification protocol (TRAP) assay using Telo TAGGG Telomerase PCR ELISA kit (Roche, Mannheim, Germany) according to the manufacturer’s recommendations. Cell extract was prepared from 50 cryostat muscle sections of 10 μm by adding 200 μl of a lysis reagent. First 1 μg and then 0.1 μg of cell lysates were used for each assay. The procedure was modified to exclude the presence of PCR inhibitors by adding phenol/chloroform isoamyl alcohol (CIA) extraction after TS extension (Hansson et al., 2008). Briefly, 2 μg of cell lysate was incubated with reaction mixture containing telomerase substrate at 25 °C for 30 min. After primer elongation, template DNA was purified by phenol/CIA extraction, before the performance of PCR. The PCR conditions were 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s. Hybridization and enzyme-linked immunosorbent assay of PCR products were done according to the supplier’s instruction. The relative telomerase activity (RTA) within different samples was calculated using the following formula:

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RTA = (A_{\text{sample}}/A_{\text{sample,IS}})/(A_{\text{TSS}}/A_{\text{TSS,IS}}) \times 100%,
\]

where \( A_{\text{sample}} \) = absorbance of sample; \( A_{\text{sample,IS}} \) = absorbance of internal standard of sample; \( A_{\text{TSS}} \) = absorbance of control template; and \( A_{\text{TSS,IS}} \) = absorbance of internal standard of control template.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.0 software. Results are expressed as mean ± standard deviation (SD). Multiple statistical comparison between groups was performed by ANOVA test, with Bonferroni’s t test post hoc correction for allowing a better evaluation of intra- and inter-group variability. The relationship between variables was studied using Pearson’s correlation test. A level of significance of \( p < 0.05 \) was considered.

3. Results

Mean telomere length values were not significantly different between DMD patients and controls (Fig. 1). Conversely, minimum telomere length values were significantly shorter in the older DMD patients versus controls (\( p < 0.004 \)) (Fig. 1). Minimum telomere length in all 20 DMD patients resulted inversely correlated to age (\( r = -0.45; p < 0.05 \)). None of the 9 younger DMD patients (≤2.5 years of age) and 4 out of the 11 older DMD patients (≥4 years of age) exhibited a minimum telomere length lower than the mean value −2SD found in control subjects.

Fig. 1. Mean and minimum telomere lengths in vastus lateralis muscles of controls and DMD patients. Mean telomere length values are not significantly different among controls and DMD subgroups according to age. On the contrary, the minimum telomere lengths of older DMD patients are significantly shorter than those of control group. Top: representative autoradiogram. * \( p < 0.004 \).
Western blot analysis revealed a 3-fold increase of TRF1 expression in the younger and in the older DMD patients ($p < 0.0001$ vs. control muscles) (Fig. 2A). PARP1 expression showed more than a 4-fold increase in both DMD patients subgroups ($p < 0.0001$ vs. control muscles) (Fig. 2B).

Real-time PCR revealed that TRF1 mRNA was increased 5.1-fold in the muscles belonging to younger DMD patients and 3.7-fold in the older DMD patients ($p < 0.002$) relative to muscles from controls (Fig. 3). PARP1 mRNA was increased 5.2-fold in the younger DMD patients and 4.6-fold in the older DMD patients ($p < 0.003$) (Fig. 3). There was no significant difference in TRF1 and PARP1 mRNA levels between the two DMD patient groups.

hTERT mRNA was increased 2.8-fold in the younger DMD patients and 2.9-fold in the older DMD patients muscles ($p < 0.02$) as compared to normal control values (Fig. 4). hTERT activity was increased in the DMD groups 1.6-fold and 1.7-fold ($p < 0.001$) respectively (Fig. 4). There was no significant difference in hTERT mRNA level and activity between the two DMD patient groups.

4. Discussion

Enhancement of the exhaustible regeneration is one of the most promising therapeutic approaches in DMD. This has been recently obtained in mdx mice through decreased inflammation by NF-kappaB (NF-κB) inhibition (Messina et al., 2006a,b, 2009; Hnia et al., 2008), modulation of insulin-like growth factor I signaling (Schertzer et al., 2007) and delivery of vascular endothelial growth factor using recombinant adenoassociated virus vectors (Messina et al., 2007). One of the possible mechanisms leading to a premature senes-
cience of satellite cells, inefficient regeneration, and then muscle loss and fibrosis has been suggested to be the reduced length of the muscle telomeric DNA. Our study confirms that minimum telomere length is shortened in DMD muscles in an age-dependent manner (Decary et al., 2000) and, in accordance with previous evidence, mean telomere length appears to be less sensitive in detecting telomere shortening (Oxelé et al., 1997). An important co-determining factor of telomere loss can be also oxidative stress (von Zglinicki, 2002), which is involved in DMD pathogenesis and represents one of the mechanisms activating NF-κB and the consequent pathogenetic cascade (Messina et al., 2006a, 2009).

We demonstrated for the first time that TRF1 and PARP1 are overexpressed in DMD muscles, suggesting their involvement in the telomere shortening. Although never investigated in skeletal muscle disorders, TRF1, PARP1 and other telomere-associated proteins have been extensively studied in cell homeostasis and pathological cell growth including cancer. Despite intense research, however, much remains unknown about the exact interactions and subsequent molecular mechanisms. It is common opinion that understanding the different processes involved in telomere maintenance, repair and stabilization may be of great help in fighting human cancer and other age-related diseases (De Boeck et al., 2009).

TRF1 overexpression in DMD muscles could be induced by oxidative stress, since it has been demonstrated that treatment of human gastric cancer cells with arsenic trioxide, which generates reactive oxygen species and oxidative stress, causes upregulation of TRF1 and TRF2 (Zhang et al., 2005). Accumulation of TRF1 at chromosome ends inhibits telomere elongation (van Steensel and de Lange, 1997), most likely contributing to satellite cell senescence. From a therapeutic point of view, our finding of TRF1 overexpression in DMD muscle is important because it leads to the identification of a novel pharmacological target. Inhibition of TRF1 can be performed by modulation of its activating kinase CK2 which has recently attracted attention for the development of anti-tumor compounds already available for in vitro or in vivo studies (Bellon et al., 2006; Kim et al., 2008; Mazzorana et al., 2008).

PARP1 is a positive regulator of telomere length and several drugs designed to inhibit PARP1 are currently under pre-clinical or clinical development as anticancer agents (Lord and Ashworth, 2008; Rodon et al., 2009). Moreover, it has been recently proposed an important cross-talk between PARP1, oxidative stress, inflammation and immune response. PARP becomes activated in response to oxidative DNA damage (Peralta-Leal et al., 2009) and pharmacological inhibition or genetic ablation of PARP1 provide remarkable protection from tissue injury in various oxidative stress-related disease models (Aguilar-Quesada et al., 2007). Several evidences also support that PARP1 has a role in some models of inflammatory disease, where its absence or inactivation confers protection, by inhibiting myeloperoxidase, cyclooxygenase-2 (COX-2), prostaglandin E2, interleukin-6 and -1beta, and NF-κB binding activity, suggesting that PARP1 inhibitory treatment can be useful in the corresponding human diseases (García et al., 2006, 2008; Sánchez-Fidalgo et al., 2007; Mota et al., 2007). Our data on an increased expression of PARP1 in DMD muscles suggest that it could represent a positive response in an attempt to allow access of telomerase to telomeres, allowing telomere elongation. However, increased PARP1 may aggravate oxidative stress damage and enhance an inflammation cascade, involving NF-κB and COX-2, already shown to contribute to dystrophic process (Messina et al., 2006a,b, 2009).

Telomerase expression and activity are detected in the majority of tumors with a 2 to 20-fold induction, but are absent in most somatic tissues and are now considered as an effective marker in cancer diagnosis and prognosis (Kim, 1997; Ghaffari et al., 2008; Hashimoto et al., 2008). The expression of the telomerase catalytic subunit hTERT in normal human myoblasts was sufficient to yield telomerase activity and elongate telomeres, but it failed to produce immortalization (Di Donna et al., 2003). The introduction of the telomerase gene in DMD myoblasts previously transformed by SV40 large T antigen considerably extended their lifespan, but still did not lead to immortalization (Seigneurin-Venin et al., 2000). In the present study we found mRNA expression of telomerase in normal skeletal muscle with detectable levels of telomerase activity, confirming the existence of a telomerase-dependent mechanism. DMD muscles exhibited a low increase of telomerase mRNA expression (almost 3-fold) and activity (less that 2-fold), which most likely are not able to maintain telomere elongation.

In conclusion, we postulate the following scheme of events. A significant telomere shortening occurs in DMD muscle, most likely due to increased degeneration-regeneration cycles and augmented oxidative stress, and can account for the declining regenerative ability of satellite cells. Telomerase pathway is activated but unable to elongate telomeres. TRF1 overexpression, induced by oxidative stress, represses telomere access to telomerase and inhibits telomere elongation. On the other hand, PARP1 overexpression can be related to occurrence of oxidative stress and be a positive compensatory response to telomere shortening, in
the attempt to inhibit TRF1 and to allow access of telomerase to telomeres. However, PARP1 activation may induce a vicious cycle, exacerbating oxidative stress-mediated injury and reinforcing the inflammatory cascade through a NF-kB-dependent mechanism. Speculatively, modulation of these events by TRF1 or PARP1 inhibitory agents might represent a novel strategy for treatment of DMD and other muscular dystrophies to counteract replicative senescence of satellite cells.

Conflict of interest

We disclose no actual or potential conflicts of interest.

References


