Telomerase Plays a Pivotal Role in Collateral Growth Under Ischemia by Suppressing Age-Induced Oxidative Stress, Expression of p53, and Pro-Apoptotic Proteins

Collateral Growth Related to Telomerase

Tomoki Kokubun, MD, Shu-ichi Saitoh, MD, Shunsuke Miura, MD, Takafumi Ishida, MD and Yasuchika Takeishi, MD

Summary

Aging is not only a major risk factor for impaired collateral growth under ischemia but also shortens the telomere length, which is regulated by telomerase. We examined the role of telomerase activity during impaired collateral growth during aging in ischemic skeletal muscle. Unilateral hind limb ischemia was generated in old, young, and old mice chronically administered a telomerase activator. In old mice, blood flow recovery and capillary density development in ischemic hind limbs were reduced compared to those in young mice, and these changes were restored to equal levels by administration of TA-65, a telomerase activator. During the early phase of ischemic muscle changes in old mice, telomerase reverse transcriptase expression and telomerase activity were both low compared to those in young mice and old mice treated with TA-65. Levels of reactive oxygen species (ROS), DNA double-strand breaks, and expression of p53, p16, and Bax/Bcl-2 were all elevated in ischemic muscles of old mice compared to those in the muscles of young mice and old mice treated with TA-65 treatment; these factors were maintained at low levels equivalent to those seen in young mice during the experiment. Expression of HIF1α/vascular endothelial growth factor (VEGF) and PGC1α were decreased in old mice compared to those in young mice and old mice treated with TA-65. Collateral growth under ischemic conditions is impaired in aged animals due to low telomerase activity, increased ROS, resultant DNA damage, and expression of tumor suppressor and pro-apoptotic proteins. These data suggest that telomerase activation enhances collateral growth and rescues ischemic tissue in old individuals.

Key words: Aging, Telomerase activity, Ischemic artery disease

Senescence is a major risk factor for cardiovascular disease, including peripheral artery disease, and impaired angiogenesis occurs with aging.1,2 A previous report demonstrated that recovery of blood flow after hind limb ischemia is impaired in older mammals compared to younger mammals.3 However, the mechanisms underlying this phenomenon remain unclear.

Telomeres are chromatin structures composed of repetitive, noncoding DNA sequences (TTAGGG in humans) at the ends of chromosomes that protect this region from degradation and recombination.4 Telomerase is a ribonucleoprotein composed of a catalytic subunit, telomerase reverse transcriptase (TERT), and a telomerase RNA template component that prevents telomere shortening by adding telomeric DNA repeats to the ends of chromosomes.5 Telomere length is maintained by telomerase complex in normal stem cells; however, it gradually shortens with age in most somatic cells where telomerase is either absent or expressed at low levels. Telomere shortening can have a critical impact on stem cell proliferation, consequently inducing vascular cell senescence.5,6 On the other hand, apart from telomere elongation, accumulating evidence indicates that telomerase plays a role in telomere-independent mechanisms involved in vascular aging, including in cellular proliferation, gene expression regulation, and mitochondrial function.5-7 However, the role of telomerase during impaired collateral flow recovery in aging after hind limb ischemia remains unclear. Therefore, we performed the present study to examine whether telomerase activation improves blood flow after acute hind limb ischemia in aged mice.

In this study, limb perfusion recovery in old mice treated with TA-65 was observed and compared to young, 8-week-old (8W) mice and old, 80-week-old (80W) mice. TA-65 is a single, small, organic molecule that is an extract from Astragalus membranaceus plant root, which is...
commonly used in traditional Chinese medicine and has been identified as an effective telomerase activator.\textsuperscript{8,11} A previous study reported that TA-65 treatment of mice not only led to a 10-fold increase in TERT mRNA and protein level in a liver and other tissue, but also increased miRNA levels of Jun B and c-Myc, two transcription factors regulated by the Mitogen-activated protein kinase (MAPK) pathway, a known potential mediator of TA-65 action. Therefore, TA-65 dependent-telomerase activation occurs through transcription factors regulated by the MAPK pathway, which may directly or indirectly regulate the nTERT promoter.\textsuperscript{4,5} We demonstrate a pivotal role for telomerase activation in angiogenesis after acute hind limb ischemia in old mice that occurs by suppressing age-related ROS and DNA damage and, subsequently, suppressing p53, p16, and apoptotic protein expression.

Methods

Animals: This study conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication, 8th edition, 2011). Our research protocol was approved by the Fukushima Medical University Animal Research Committee [permit number 29074 (24 July 2017)], and all animal experiments were conducted in accordance with the guidelines of the Fukushima Medical University Animal Research Committee. All efforts were made to minimize animal suffering. Young and old wild-type C57BL/6 male mice [young: 8 weeks (W); body weight (BW), 18.4 ± 0.2 g; old: 80W; BW, 29.3 ± 0.5 g] were housed and bred in a room maintained at 22 ± 3°C with a relative humidity of 50 ± 10% and a 12-hour light-dark cycle. Mice were given water \textit{ad libitum}.

Animal experiments: Young mice were given saline, and old mice were administered either saline or the telomerase activator TA-65 (T.A. Sciences, Inc. NY, USA). In reference to doses used in past papers, TA-65 was dissolved in activator TA-65 (T.A. Sciences, Inc. NY, USA). In refer-

Hindlimb ischemia: The left femoral artery was exposed under tribromoethanol anesthesia (200 mg/kg/body weight, i.p.) and was ligated at two positions, just below the inguinal ligament and proximal to the saphenous-popliteal bifurcation using 6-0 sutures. Sham operations occurred without femoral artery ligation but with a skin incision made in the right hind limb.

Hind limb perfusion measurement: Scanning laser Doppler perfusion imaging (Moor Instruments, Wilmington, DE, USA) was used to measure hind limb perfusion under 1.125% isoflurane/O2 anesthesia. The ratio of ischemic/ non-ischemic laser Doppler blood flow was measured before and on postoperative days 0, 1, 7, 14, and 21.

Reactive oxygen species (ROS) measurement: An Ox-iSelect\textsuperscript{TM} In Vitro ROS/RNS Assay Kit (Green Fluorescence) was used to measure levels of ROS according to the protocol provided by the manufacturer (Cell Biolabs, Inc. San Diego, CA, USA). Twenty milligrams of harvested soleus muscle tissues were cut to pieces 5 mm away from the Achilles tendon and homogenized in 1 mL of ice-cold PBS. Homogenized samples were centrifuged at 10,000 g for 5 minutes to remove insoluble particles. The supernatant of each sample was used. We added 50 μL of each sample in duplicate and hydrogen peroxide (H2O2) standards to a black 96-well plate with 50 μL of the provided catalyst solution and incubated the mixture for 5 minutes at room temperature to accelerate the oxidative reaction. Next, we added 100 μL of dichlorodihydrofluorescein (DCFH) solution included in the kit to each well, covered the plates to protect them from light, and incubated the samples at room temperature for 30 minutes. Finally, fluorescence was read with a fluorescence plate reader (SpectraMax\textsuperscript{i3, Molecular Devices}) at 480 nm excitation/530 nm emission. A H2O2 standard curve was used to quantify total free radical content in skeletal muscle.

Telomerase activity measurement: The telomerase repeated amplification protocol (TRAP) assay is a popular method for determining telomerase activity in mammalian cells and tissue samples.\textsuperscript{14} Telomerase activity was measured using the Telo TAGGG Telomerase PCR ELISA\textsuperscript{PLUS} Assay Kit (Cat. No. 12013789001, Roche Diagnostics GmbH, Mannheim, Germany), a photometric enzyme immunoassay for quantitative determination of telomerase activity that uses TRAP. Tissues extracts were prepared from frozen tissue pellets stored at ~80°C by adding 200 μL of lysis reagent according to the manufacturer’s instructions. The lysis mixture was homogenized and incubated on ice for 30 minutes. The lystate was then centrifuged at 16,000 x g for 20 minutes at 4°C, and the supernatant was collected. We measured protein concentrations by standard methods and then performed the TRAP reaction as described below.

First, negative controls for each sample were prepared by heating at 95°C for 10 minutes. Then, we prepared 30 μL of master mix (25 μL of reaction mixture, 5 μL of the provided internal standard) per sample. We constructed 50 μL reaction mixtures for each sample (30 μL of master mix, X μL of sample per 10 μg of total protein and added nuclease free water for a total volume of 50 μL), negative controls (30 μL of master mix, X μL of heat-treated sample per 10 μg of total protein and added nuclease free water for a total volume of 50 μL), and control templates (30 μL of master mix, with 1 μL of control template added to a separate tube, 1 μL of lysis reagent added to another tube and 19 μL of nuclease free water added to each tube), all of which were added to a new 0.2-mL tube suitable for PCR. The microtubes were transferred to a PCR thermal cycler (GeneAmp\textsuperscript{®} PCR system 9700, Applied Biosystems) for combined primer elongation/amplification reaction using the following protocol [1 cycle for 20 minutes at 25°C (primer elongation); 1 cycle for 5 minutes at 94°C (telomerase inactivation); 30 cycles for 30 seconds at 94°C, 30 seconds at 50°C, 90 seconds...
at 72°C and 1 cycle for 10 minutes at 72°C (amplification, PCR). After 2.5 μL of amplification products were mixed with 10 μL of denaturation reagent, we added 100 μL of hybridization buffer according to the supplier’s instructions. Then, we transferred the 100-μL samples to a pre-coated MP module included in the kit and incubated for two hours at room temperature with agitation at 300 rpm. Then, the MP modules were washed three times, and 100 μL of anti-DIG-HRP was added to each well and incubated for 30 minutes with agitation at 300 rpm. After washing the plates three times, 100 μL of pre-warmed TMB substrate solution was added to the wells, and the wells were incubated at room temperature for 20 minutes with agitation for color development. Then, 100 μL of stop reagent was added to each well to stop color development. Finally, we measured sample absorbance at 450 nm using a microplate reader (with a reference wavelength of approximately 690 nm). Relative telomerase activity (RTA) was calculated using the following formula:

\[
RTA = \left( \frac{A_{TS8,0} - A_{S, IS}}{A_{TS8, IS} - A_{S, IS}} \right) \times 100\%
\]

A indicates absorbance of the sample; A_{S, IS}, absorbance of heat-or RNase-treated samples; A_{TS8, IS}, absorbance of the control template (TS8); A_{TS8,0}, absorbance of the lysis buffer; and A_{S, IS}, absorbance of the IS of the Control template (TS8).

**Western blotting:** Harvested soleus muscles frozen at −80°C were pulverized in liquid nitrogen and suspended in lysis buffer with a protease inhibitor. After determining protein concentrations using a standard protocol (Bradford protein assay, Bio-Rad), equal amounts of protein (20 μg/sample) were analyzed by 7.5%, 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels depending on the molecular weight of the targeted proteins. Gels were transferred onto nitrocellulose membranes that were blocked with tris-buffered saline (5% BSA) and immunoblotted using the following primary antibodies at 4°C with gentle agitation overnight: anti-p53 (PAb122) (Enzo Life Sciences, Inc., Farmingdale, NY, USA), anti-p21 (sc-6246) (Santa Cruz Biotechnology, Inc. 1:1000), anti-p53 (PAb122) (Enzo Life Sciences, Inc., Farmingdale, NY, USA). Data were normalized to β-Actin and are expressed as fold change to the non-ischemic skeletal muscle of young mice in the same period for each analysis.

**Immunohistochemistry:** Capillary formation in ischemic soleus muscles was analyzed by immunostaining for CD31 and quantified as capillaries per muscle fiber. Immunohistochemistry was performed using paraffin-embedded tissue (HIC-P). Capillary counts were performed under light microscopy (200 ×). The number of capillaries was measured in five randomly chosen fields from three different sections in each tissue block.

**Statistical analysis:** All values are expressed as the means ± SEM. One-way repeated measures ANOVA with Tukey’s post hoc test used to compare hind limb blood flow data. For all other data sets, one-way factorial ANOVA with Tukey’s post hoc test were performed to compare multiple groups. P < 0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS software (ver. 24.0, IBM, Armonk, NY, USA).

**Results**

**Limb blood flow recovery:** Blood flow recovery was impaired in ischemic hind limbs of old mice compared to those of young mice (Figure 1A and B). TA-65 treatment in old mice improved blood flow recovery. Histologically, TA-65 treatment also increased capillary density as measured by CD31 staining of muscle tissue in old mice, which reflected improved blood flow recovery (Figure 1C and D). These data support the notion that collateral flow recovery under ischemic conditions is impaired in aging, and telomerase activation by TA-65 treatment could improve age-related angiogenesis impairment.

**Levels of ROS in skeletal muscle tissue:** Oxidative stress increasing with age, and high ROS levels are cytotoxic, impairing angiogenesis. We previously reported that age-related increases in ROS levels after hind limb ischemia impair limb blood flow recovery. We also demonstrated that mitochondrial ROS scavenging in old mice contributes to collateral flow recovery after hind limb ischemia. Therefore, we initially measured ROS levels in non-ischemic and ischemic soleus muscles at POD 3. No difference was found among non-ischemic animals (Figure 2A). ROS levels in old mice’s ischemic limbs were elevated compared to those in non-ischemic regions and were prominent compared to those in young mice’s limbs. Furthermore, ROS levels were attenuated by TA-65 treatment in old mice. These findings suggest that ROS levels increase in aging tissue under ischemia and are attenuated by TA-65 treatment.

**DNA damage in skeletal muscle tissue:** ROS, telomere dysfunction, UV exposure, etc., cause DNA damage responses (DDRs), particularly double-strand breaks (DSBs); DSBs are the most serious type of DNA damage and induce apoptosis and cellular senescence, resulting in vascular aging. DSBs trigger activation of many factors, including phosphorylation of histone variant H2AX (γH2AX), which plays an important role in the DDR and...
is required to assemble DNA repair proteins at sites containing damaged chromatin and to activate checkpoint proteins that cause cell cycle arrest. Therefore, γH2AX is a useful tool for detecting DNA damage. To evaluate DNA damage in response to hind limb ischemia, we measured γH2AX expression in soleus muscle by Western blotting. On POD 3 and POD 21, γH2AX expression increased in ischemic limbs compared to non-ischemic limbs (Figure 2 B). In ischemic muscles, the expression levels of γH2AX were high in old mice compared to young mice and were
Figure 3. TERT expression and telomerase activity. On postoperative day (POD) 3, TERT expression (A) and telomerase activity (B) were elevated under conditions of ischemia compared to non-ischemic conditions. In ischemic groups on POD 3, TERT expression levels and telomerase activity in old mice were lower than those in young mice and were upregulated in old mice with TA-65 treatment. Data are expressed as the means ± SEM. n = 18, each. *P < 0.05 versus young mice. $P < 0.05 versus old mice. #P < 0.05 versus non-ischemic muscle.

TERT expression and telomerase activity: Telomerase’s enzymatic activity has been proposed to be primarily controlled by transcriptional regulation of TERT. TERT gene transfer into endothelial progenitor cells contributed to improvement in angiogenesis in a murine model of hind limb ischemia.20) Thus, impaired TERT expression and telomerase activity may contribute to impaired angiogenesis in the elderly. We measured TERT expression and telomerase activity to investigate the effects on aging in response to ischemia and to confirm telomerase activation by TA-65. On POD 3, TERT expression and telomerase activity were significantly elevated in ischemic limbs compared to non-ischemic limbs (Figure 3A and B). In ischemic muscles, TERT expression and telomerase activity in old mice were reduced compared to those in young mice and were upregulated in old mice by TA-65 treatment. In contrast, on POD 21, TERT expression was higher in old mice than in young mice, and the same tendency was found in TERT levels between young mice and old mice treated with TA-65. TA-65 treatment regulated TERT levels at POD 21 and POD 3. In contrast, the effect of TA-65 on telomerase activity at POD 21 in ischemic limbs in old mice was negligible. It has been reported that TERT expression is upregulated in response to cellular stress, such as that caused by ischemia.21) On POD 21, blood flow recovery in ischemic hindlimbs of young and TA-65-treated old mice were improved to nearly the same level as in non-ischemic mice, as described above. Therefore, we proposed that there was a reduced influence of hypoxia and ischemia in young mice and in old mice treated with TA-65 during the late phase of ischemia. These data suggest that TERT expression and telomerase activity that were upregulated in response to ischemia were attenuated in the elderly, especially during the early phase of hindlimb ischemia, and were recovered after TA-65 treatment.

Expression of p53/p21, p15/16 and apoptotic factors: Telomere shortening, or DNA damage, induces DDR. When the damage becomes irreversible, the DDR pathway induces cellular senescence, cell cycle arrest and apoptosis. The transcription factor p53, also known as the tumor suppressor gene, is widely recognized as one of the downstream effectors in the DDR pathway that plays an important role through activating pathways via post-translational modifications, such as acetylation or phosphorylation. Considering our findings that TA-65 decreased ROS levels and DNA damage, we assumed that TA-65 treatment would attenuate p53 expression. Thus, we examined whether TA-65 treatment downregulated p53 or p21 expression in ischemic aged skeletal muscles. In non-ischemic groups, p53 expression on POD 3 and POD 21 in old mice tended to be higher than in young mice and TA-65 treated mice (Figure 4A). p53 expression was higher in ischemic old mice than in non-ischemic mice and was attenuated by treatment with TA-65 (Figure 4B). Expression of p21 showed the same tendency, and a similar trend was recognized in p53/p21 expression on POD 3 and POD 21 (Figure 4C). The p16/RB (retinoblastoma protein) pathway is also another major pathway of senescence. Aging and many inducers of cellular senescence, such as ROS, increase p16 activity.22) We measured p16 expression at POD 3 and POD 21, and found that expression was higher in ischemic old mice than in non-

attenuated in old mice treated with TA-65, resulting in levels equal to those in young animals. These results suggest that ischemia-induced DNA damage in skeletal muscle increases with aging and is suppressed by TA-65 treatment.
Figure 4. Expression of p53, p21, p16, and apoptotic factors. Representative Western blots shown (A). p53 (B) and p21 (C) expression levels were higher in ischemic old mice than in non-ischemic groups and were attenuated in old mice by administration of TA-65. On postoperative day (POD) 3 and 21, p16 (D) expression levels and the Bax/Bcl-2 ratio (E) were higher in ischemic old mice than in non-ischemic mice. In ischemic groups, p16 expression levels and the Bax/Bcl-2 ratio in old mice were higher than those in young mice and were attenuated in old mice by administration of TA-65. Data are expressed as the means ± SEM. n = 18, each, *P < 0.05 versus young mice. $P < 0.05 versus old mice. #P < 0.05 versus non-ischemic muscle.
ischemic limbs and decreased in TA-65-treated old mice (Figure 4D). These findings suggest that a decline in ROS and subsequent DNA damage with TA-65 treatment in aged skeletal muscle attenuate p53/p21 and p16 expression in response to ischemia. Apoptosis, a programmed form of cell death, is considered an anti-angiogenic mechanism.20 Several studies have reported that telomerase activity is essential for endothelial cell differentiation and protection from apoptosis.20 The B-cell leukemia/lymphoma-2 (Bcl-2) family of proteins are the best-characterized protein family involved in regulating apoptotic cell death. It includes anti-apoptotic and pro-apoptotic members. Activated p53, in response to DNA damage, mediates mitochondrial control of apoptosis by promoting pro-apoptotic members (Bax) and suppressing anti-apoptotic Bcl-2.21 Therefore, we examined whether telomerase activation prevents apoptosis under ischemia conditions. The Bax/Bcl-2 ratio was higher in ischemic old mice than in young mice, and TA-65 treatment of old mice decreased the Bax/Bcl-2 ratio to that of young mice at both POD 3 and POD 21 (Figure 4E). These data suggest that telomerase activation by TA-65 is protective against ischemia-induced apoptosis in aged skeletal muscle and contributes to angiogenesis.

**HIF-1α-VEGF signaling pathways:** Ischemia-induced angiogenesis is impaired in aging and is mediated by hypoxia inducible factor-1 (HIF-1α).26 HIF-1α is a transcription factor that mediates adaptive responses to hypoxia and ischemia. HIF-1α regulates expression of angiogenic factors, such as VEGF, via the HIF-1α-VEGF signaling pathways.27 p53 is known as a potent negative regulator of HIF-1α and VEGF during hypoxia.28 To examine TA-65 treatment's angiogenetic effects, we measured expression of HIF-1α and VEGF by Western blot analysis (Figure 5A). At POD 3, expression of HIF-1α and VEGF were significantly higher in young mice than non-ischemic mice (Figure 5B and C). This elevation was suppressed in old mice and recovered with TA-65 treatment. However, on POD 21, expression of HIF-1α was not different among the three groups. VEGF expression in young mice was higher than that in old mice, and there was a tendency for increased expression of VEGF in old mice treated with TA-65 compared to those that were not treated, though this change did not reach statistical significance. These data suggest that telomerase activation by TA-65 is associated with elevated HIF-1α and VEGF expression.

---

**Figure 5.** Expression of HIF-1α, VEGF, and PGC-1α. On postoperative day (POD) 3 after induction of ischemia, expression of HIF-1α and VEGF were increased in young mice compared to non-ischemic mice (A-C). These elevations were suppressed in old mice and upregulated by TA-65 treatment. However, on POD 21 after induction of ischemia, expression of these factors were not different among the three groups. Furthermore, in ischemic skeletal muscles on POD 3, expression of PGC-1α was reduced in old mice compared to young mice, and TA-65 treatment preserved PGC-1α to a similar level as in young mice (D). However, there were no differences in PGC-1α expression in ischemic skeletal muscle on POD 21. A: Representative Western blots. B-D: Summarized data. Data are expressed as the means ± SEM. n = 18, each. *P < 0.05 versus young mice. $P < 0.05 versus old mice. #P < 0.05 versus non-ischemic muscle.
expression in aged skeletal muscle at comparatively early phases of ischemia. On POD 21, blood flow recovery in ischemic hind limbs of young mice and TA-65-treated old mice improved to nearly the same level as in non-ischemic mice described above. Therefore, we propose that hypoxia and ischemia exert less of an influence in young mice and in old mice treated with TA-65 than in untreated old mice during the late phase of ischemia.

Expression of peroxisome proliferator activated receptor γ coactivator 1α (PGC-1α): Recent studies have reported that telomere dysfunction, and associated DNA damage responses, promote mitochondrial dysfunction.29) p53 induced by telomere dysfunction binds to the PGC-1α promoter and suppresses PGC-1α expression. PGC-1α repression impairs mitochondrial biogenesis and function, resulting in reduced ATP generation and increased ROS levels.30) In light of our previous experiments illustrating that telomerase activation by TA-65 attenuates ROS and DNA damage along with decreasing p53 levels in aged ischemic skeletal muscle, we assumed that TA-65 treatment would preserve PGC1α expression under conditions of ischemia. Expression of PGC-1α did not differ in the non-ischemic skeletal muscles at POD 3 or POD 21. In ischemic skeletal muscles at POD 3, expression of PGC-1α was significantly lower in old mice than in young mice, and TA-65 treatment of old mice maintained PGC-1α levels at similar levels as those in young mice (Figure 5D). However, there were no differences in PGC-1α expression in ischemic skeletal muscle at POD 21. Considering the reduction in ROS, protection from apoptosis, and preservation of PGC-1α expression by TA-65 treatment in ischemic aged mice, these data suggest that telomerase activation improves mitochondrial dysfunction in aged mice after hind limb ischemia, especially during early phases of ischemia.

Discussion

Senescence is a dominant risk factor for most forms of cardiovascular disease.31) The incidence of peripheral artery disease (PAD) is currently increasing. Critical limb ischemia is an end-stage of PAD and is associated with a high risk of lower limb loss.32) Therefore, new treatments that rescue the ischemic limb for patients of PAD are necessary. Telomerase is closely related with cellular senescence and may be a potential anti-aging therapy.33) Our present study demonstrates the crucial role of telomerase activity for improving blood flow after acute hind limb ischemia in old mice. Telomerase activation therapy in aged skeletal muscle suppresses age-related ROS and DNA damage in response to ischemia, contributing to angiogenesis through the tumor suppressors p53 and p16 and pro-apoptotic proteins (Figure 6).

The tumor suppressors p53 and p16 are upregulated in response to various cellular stresses, including DNA damage, oxidative stress and hypoxia.35,34,36) Upregulating p53 induces dramatic and multiple cellular responses, including apoptosis and anti-angiogenesis via the HIF-1α/VEGF pathway.37,38) In the present study, as in our previous study, p53 was upregulated by aging and in response to ischemic DNA damage, which seemed to promote apoptotic processes and inhibit angiogenic processes. Telomerase activation markedly downregulated p53 expression in response to ischemia. Considering that DDR following DNA damage is the most important pathway for p53 upregulation, reduction of ROS and DNA damage by
The mitochondria. Telomerase activation under ischemia conditions may contribute to angiogenesis, not only through p53 downregulation but also through p16 reduction, resulting in collateral flow recovery.

Furthermore, apoptosis is considered an anti-angiogenic mechanism, and p53 activated due to cellular stress mediates mitochondrial control of apoptosis. Considering the observed downregulation of p53 and the Bax/Bcl-2 ratio in ischemic muscle, telomerase activation might contribute to inhibiting apoptosis, resulting in angiogenesis and collateral flow recovery. In this study, we examined apoptosis in isolated muscle, including vessel, because it is difficult to isolate the capillary. Therefore, further study is needed, like an in vitro study, to clarify TA-65’s pure contribution to angiogenesis and apoptosis.

It has been proposed that the enzymatic activity of telomerase is primarily controlled by transcriptional regulation of TERT, as shown in our results at POD 3. In contrast, there are many reports that telomerase activity is regulated by several factors, such as ROS, exercise capacity and cellular stress related to the magnitude of ischemia, which may affect TERT expression and telomerase activity in chronic ischemic conditions. We did not observe a positive correlation between telomerase activity and TERT expression in ischemic old mice at POD 21. Therefore, further study is needed to investigate the effect of telomerase on various conditions, including acute and chronic ischemic states. TERT has also been proposed to play a role in regulating oxidative damage-induced apoptosis. Oxidative stress triggers nuclear export of TERT to the mitochondria. TERT has been shown to localize to mitochondria and contributes different mitochondrial functions (such as modulating mtDNA integrity, improving respiratory chain function and affecting ROS production). Therefore, we believe that the main source of ROS is mitochondria. As previously presented, the chronic scavenging of superoxide, which is dropped from mitochondria ETc-complex in aged skeletal muscle like a condensation of attenuated telomerase activity, improved collateral growth induced by ischemia through regulation of p53 as a mimicking effect of TA-65 treatment. Therefore, mitochondria ETc-complex activity or drop of superoxide from ETc-complex pathway might be modified by telomerase activity and TA-65 decrease generation of superoxide in ETc-complex in mitochondria, though further study is needed to clarify the telomerase effect to mitochondria. Thus, antioxidant effect of telomerase or TA-65 could improve mitochondrial dysfunction and contribute to angiogenesis through preservation of PGC-1α in aged skeletal muscle after hind limb ischemia. There are a few limitations to this study. First, we did not measure telomere length in this study. Evidence of telomerase’s additional functions, other than telomere maintenance, have been accumulating recently. Human adult somatic cells do not express telomerase activity, whereas most adult mouse somatic cells, including skeletal muscle, express detectable amounts of telomerase. In fact, it has been reported that there is no significant difference in telomere length between young and aged muscle stem cells, known as satellite cells, from uninjured wild-type mice. Considering the lack of differences in telomere length in response to aging in the skeletal muscles of mice, the rapid onset of the anti-apoptotic process and angiogenic differences in old mice treated with TA-65 detectable at day three in vitro seems to depend on telomerase rather than on telomere length. Second, we evaluated only two time points, POD 3 and POD 21 (reference our previous study). It is presumed that signal transduction, including TERT, may change dramatically, especially during the early phase of ischemia, but we did not demonstrate sequential changes. Third, we did not perform measurements in muscles composed of fast-twitch muscle fibers, such as the gastrocnemius. It has been reported that age-related mitochondrial function, one of the important targets of telomerase, varies in different muscle types. Thus, further study is needed to investigate the telomerase performance in other muscle types.

Conclusion

We showed a possible role for telomerase in regulating cellular processes during senescence other than telomere elongation, such as differentiation and angiogenesis, through tumor suppressors p53 and p16. Our data suggest that telomerase plays a pivotal role in collateral development, and its pharmaceutical activation may be a novel therapeutic option to enhance collateral flow recovery to rescue ischemic tissue in elderly individuals.

Acknowledgments

We thank Tomiko Miura and Chikako Endo for the care of the mice and for laboratory assistance.

Disclosures

Conflicts of interest: None.

References

1. WRITING GROUP MEMBERS, Lloyd-Jones D, Adams RJ, et al. Heart disease and stroke statistics--2010 update: a report from the American Heart Association. Circulation 2010; 121: e86-215.
2. Soga Y, Iida O, Takahara M, et al. Two-year life expectancy in patients with critical limb ischemia. JACC Cardiovasc Interv 2014; 7: 1444-9.
3. Rivard A, Fabre JE, Silver M, et al. Age-dependent impairment of ischemic muscle angiogenesis by treatment with telomerase.
of angiogenesis. Circulation 1999; 99: 111-20.
4. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 1985; 43: 405-13.
5. Rodier F, Campisi J. Four faces of cellular senescence. J Cell Biol 2011; 192: 547-56.
6. van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. Cell 1998; 92: 401-13.
7. Hartwig FP, Nedel F, Collins SR, Tarquinio SB, Nör JE, De Marco FF. Telomeres and tissue engineering: the potential roles of tert in VEGF-mediated angiogenesis. Stem Cell Rev 2012; 8: 1275-81.
8. Fu W, Begley JG, Killen MW, Mattson MP. Anti-apoptotic role of telomerase in pheochromocytoma cells. J Biol Chem 1999; 274: 7264-71.
9. Bernardes de Jesus B, Schneeberger K, Vera E, Tejera A, Harley CB, Blasco MA. The telomerase activator TA-65 elongates short telomeres and increases health span of adult/old mice without increasing cancer incidence. Aging Cell 2011; 10: 604-21.
10. Tel, Liu W, Blasco M, et al. A natural product telomerase activator as part of a health maintenance program. Rejuvenation Res 2011; 14: 45-56.
11. Salvador L, Singaravelu G, Harley CB, Flom P, Suram A, Rafaele JM. A natural product telomerase activator lengthens telomeres in humans: A randomized, double blind, and placebo controlled study. Rejuvenation Res 2016; 19: 478-84.
12. Mouraret N, Housainti A, Abid S, et al. Role for telomerase in pulmonary hypertension. Circulation 2015; 131: 742-55.
13. Miura S, Saitoh SI, Kokubun T, et al. Mitochondrial-targeted antioxidant maintains blood flow, mitochondrial function, and redox balance in old mice following prolonged limb ischemia. Int J Mol Sci 2017; 18: 1897.
14. Kim NW, Piatsyzevk M, Prowse WR, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994; 266: 2011-5.
15. Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. Free Radic Biol Med 2009; 46: 50-9.
16. Radomska-Leśniewska DM, Hevelke A, Skopinski P, et al. Telomerase and synthetic antioxidants as angiogenesis modulators: clinical implications. Pharmacol Rep 2016; 68: 462-71.
17. Yamauchi H, Miura S, Owada T, et al. Senescence marker protein-30 deficiency impairs angiogenesis under ischemia. Free Radic Biol Med 2016; 94: 66-73.
18. Hoeijmakers JH. DNA damage, aging, and cancer. N Engl J Med 2000; 342: 1031-42.
19. Inoue E, Tano K, Yoshii H, et al. Age-dependent regulation of p53 in p53-deficient human telomere-directed senescence. Curr Biol 2004; 14: 1587-91.
20. Piacentini M, Borchers J, van der Grinten R, et al. Telomerase activity is a major determinant of tumor angiogenesis: an analysis of the p53 and p16INK4a allelic status in prostate cancer. Cancer Res 2002; 62: 5684-90.
21. Zurek M, Altschmied J, Kohlgrüber S, Ale-Agha N, Haendeler J. Role of telomerase in the cardiovascular system. Genes (Basel) 2016; 7: 39.
22. Vossen NH, Prives C. Blinded by the light: the growing complexity of p53. Cell 2009; 137: 413-31.
23. Jacobs JJ, de Lange T. Significant role for p16INK4a in p53-independent telomere-directed senescence. Curr Biol 2004; 14: 2302-8.
24. Tesz G, Madalinski J, Price VN, et al. Telomerase targets specific nuclear proteins in a telomere-telomere interaction. PLoS One 2011; 6: 18317.
25. Chipuk JE, Kuwana T, Bouchier-Hayes L, et al. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 2004; 303: 1010-4.
26. Rivald A, Berthou-Soulie L, Prince N, et al. Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. J Biol Chem 2000; 275: 29643-7.
27. Bosch-Marce M, Okuyama H, Wesley JB, et al. Effects of aging and hypoxia-inducible factor-1 activity on angiogenic cell mobilization and recovery of perfusion after limb ischemia. Circ Res 2007; 101: 1310-8.
28. Ravi R, Mookerjee B, Bhujwalla ZM, et al. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev 2000; 14: 34-44.
29. Sahin E, Colla S, Liesa M, et al. Telomere dysfunction induces metabolic and mitochondrial compromise. Nature 2011; 470: 359-65.
30. Sahin E, DePinho RA. Axis of aging: telomeres, p53 and mitochodria. Nat Rev Mol Cell Biol 2012; 13: 397-404.
31. Lahteenmäki J, Rosenzweig A. Effects of aging on angiogenesis. Circ Res 2012; 110: 1252-64.
32. Akagi D, Hoshina K, Akai A, Yamamoto K. Outcomes in patients with critical limb ischemia due to arteriosclerosis obliterans who did not undergo arterial reconstruction. Int J Heart J 2018; 59: 1041-6.
33. Jäger K, Walter M. Therapeutic targeting of telomerase, Genes (Basel) 2016; 7: 39.
34. Vossen NH, Prives C. Blinded by the light: the growing complexity of p53. Cell 2009; 137: 413-31.
35. Jacobs JJ, de Lange T. Significant role for p16INK4a in p53-independent telomere-directed senescence. Curr Biol 2004; 14: 2302-8.
36. Yoshi P, MacKenzie KL, Keith WN, et al. Therapeutic targeting of replicative immortality. Semin Cancer Biol 2015; 35: S104-28.
37. Aamaral JD, Xavier JM, Steer CJ, Rodrigues CM. The role of p53 in apoptosis. Discov Med 2010; 9: 145-52.
38. Farhang Ghaemrani M, Goossens S, Nittner D, et al. p53 promotes VEGF expression and angiogenesis in the absence of an intact p21-Rb pathway. Cell Death Differ 2013; 20: 888-97.
39. Zhang J, Lu A, Li L, Yue J, Lu Y. p16 Modulates VEGF expression via its interaction with HIF-1alpha in breast cancer cells. Cancer Invest 2010; 28: 588-97.
40. Chung J, Khadka P, Chung IK. Nuclear import of hTERT requires a bipartite nuclear localization signal and Akt-mediated phosphorylation. J Cell Sci 2012; 125: 2684-97.
41. Babizhayev MA, Yegorov YE. Tissue formation and tissue engineering through host cell recruitment or a potential injectable cell-based bio composite with replicative potential. Molecular mechanisms controlling cellular senescence and the involvement of controlled transient telomerase activation therapies. J Biomed Mater Res A 2015; 103: 3993-4023.
42. Cong Y, Shay JW. Actions of human telomerase beyond telomeres. Cell Res 2008; 18: 725-32.
43. Arany Z, Foo SY, Ma Y, et al. HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. Nature 2008; 451: 1008-12.
44. Kipling D. Telomere structure and telomerase expression during human telomeres. Cell Res 2008; 18: 405-13.
45. Zarek M, Altschmied J, Kohlgruber S, Ale-Agha N, Haendeler J. Role of telomerase in the cardiovascular system. Genes (Basel) 2016; 7: E29.
46. Arany Z, Foo SY, Ma Y, et al. HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. Nature 2008; 451: 1008-12.
47. Kipling D. Telomere structure and telomerase expression during mouse development and tumorgenesis. Eur J Cancer 1997; 33: 792-800.
48. Tichy ED, Sidibe DK, Tierney MT, et al. Single stem cell imaging and analysis reveals telomere length differences in diseased human and mouse skeletal muscles. Stem Cell Res 2017; 20: 315-28.