Mechanisms of Telomerase Induction During Vascular Smooth Muscle Cell Proliferation

T. Minamino, S. Kourembanas

Abstract—Telomeres are primarily controlled by a highly specialized DNA polymerase termed telomerase. Recent studies have demonstrated that introduction of the telomerase catalytic component (TERT) into telomerase-negative cells activates telomerase and extends cell life span, whereas mice lacking telomerase activity revealed impaired cell proliferation in some organs as well as reduced tumorigenesis. These reports suggest that telomerase plays an important role in long-term cell viability and cell proliferation. However, the mechanism or mechanisms by which telomerase is induced or regulated remains to be elucidated. We report here that primary vascular smooth muscle cells (VSMCs) express telomerase and that increased telomerase activity correlates with cell proliferation. Inhibition of telomerase diminished growth of VSMCs, which suggests a crucial role for telomerase activation in the regulation of VSMC proliferation. We propose a novel model whereby telomerase is first activated in the cytoplasm before cell proliferation, followed by accumulation of activity in the nucleus during the logarithmic phase of cell growth. Activation of telomerase in VSMCs was linked to phosphorylation of TERT. The protein kinase inhibitor H7 suppressed the activation of telomerase in the cytoplasm and also inhibited the accumulation of TERT as well as telomerase activity in the nucleus. These data suggest that posttranslational modification of TERT by phosphorylation is important for activation and accumulation of telomerase into the nucleus in the process of VSMC proliferation. (Circ Res. 2001;89:237-243.)

Key Words: telomere ■ telomerase ■ vascular smooth muscle cell ■ phosphorylation ■ proliferation

Telomeres consist of repeats of the sequence TTAGGG at the end of chromosomes. These DNA repeats are synthesized by enzymatic activity associated with an RNA protein complex called telomerase.1 In most somatic cells, telomerase activity is undetectable, and telomere length decreases with increasing cell division. In contrast, in most cancer cells, telomeres stop shortening because of de novo synthesis of telomeric DNA by activated telomerase.2 Inhibition of telomerase has been shown to induce growth inhibition in cultured cancer cells.3 Thus, telomerase activation has been implicated in tumorigenesis. Recently, mice lacking telomerase RNA have been established and reported to show not only reduced tumorigenesis4 but also decreased cell proliferation in highly proliferative organs,5 which suggests that telomerase activity may be involved in the regulation of cell proliferation in normal somatic cells as well.

Mammalian telomerase consists of an RNA component (TERC) and two protein components, telomerase-associated protein 1 (TEP1) and telomerase reverse transcriptase (TERT).6–8 Expression of TERC was shown to correlate with cell proliferation as well as telomerase activity in cancer cells.6 TERT mRNA levels were also reported to correlate with telomerase activity and to be implicated in the regulation of telomerase activity in cancer cells.8 Furthermore, telomerase activity in telomerase-negative cells can be restored by ectopic expression of TERT,9 which suggests that in certain cases, TERT is the only limiting factor for telomerase activation. In addition to transcriptional regulation for telomerase activation, there is accumulating evidence that telomerase activity is also controlled by protein phosphorylation10 that may induce11 or inhibit TERT activity.12 However, the mechanism or mechanisms underlying telomerase regulation mediated by protein phosphorylation remain largely unknown.

We report here that primary vascular smooth muscle cells (VSMCs) express telomerase activity when stimulated to proliferate. We propose a novel model whereby telomerase is first activated in the cytoplasm before cell proliferation, followed by accumulation of activity in the nucleus during the logarithmic phase of cell growth. Our data indicate that both processes involve mechanisms mediated by protein phosphorylation. Moreover, TERT was found to be phosphorylated in VSMCs, implying that posttranslational modification of TERT by phosphorylation plays an important role in the regulation of telomerase activity.

Original received January 24, 2001; revision received June 8, 2001; accepted June 8, 2001.
From the Department of Medicine, Division of Newborn Medicine, Children’s Hospital and Department of Pediatrics, Harvard Medical School, Boston, Mass.
Correspondence to Stella Kourembanas, MD, Division of Newborn Medicine, Children’s Hospital, Enders 9, 300 Longwood Ave, Boston, MA 02115.
E-mail stella.kourembanas@tch.harvard.edu
© 2001 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org
Materials and Methods
The kinase inhibitors herbimycin A, tyrphostin A25, H7, myristoylated
protein kinase A (PKA) inhibitory peptide, myristoylated protein
kinase C (PKC) inhibitory peptide, protein kinase G (PKG)
inhibitory peptide, and PD98059, and the telomerase inhibitor TAG,
were purchased from Calbiochem. All other materials used were
obtained from Sigma unless otherwise indicated.

Cell Culture
Primary cultures of rat aortic VSMCs and rat fetal VSMCs and A7r5
cells were maintained as described.13

Induction of TERT-FLAG in A7r5 Cells
The expression vector, pcDNA3 TERT-FLAG, was the kind gift of
Dr F. Ishikawa (Department of Life Science, Tokyo Institute of
Technology, Japan). Transfection of A7r5 cells was performed by
Fugene (Boehringer Mannheim) according to the manufacturer’s
instructions. TERT-FLAG clones were selected in the presence of 1
mg/mL G418.

Preparation of Cell Extracts and Telomerase
Activity Measurement
Cytoplasmic and nuclear extracts were prepared as described.14 Telomerase activity was assayed by stretch polymerase chain
reaction (PCR) assay as described previously.15 The modified telomeric
repeat amplification protocol (Oncor) was also used for telomerase
activity assay and performed according to the manufacturer’s in-
structions.16 To confirm the linearity of the stretch PCR assay, we
performed pilot experiments for each extract. According to these
experiments, 4×10^3 cell equivalents were subjected to stretch PCR
assay. The specificity of telomerase products was determined by
their sensitivity to preincubation with RNase for each sample.
Telomerase products were quantified using an image analyzer
(Molecular Dynamics).

RNA Analysis
Total RNA samples were extracted using RNAzol B (Teltest). To
examine TERC expression, Northern blot analysis using 30 µg of
RNA was performed. The blot was probed with a cRNA probe under
highly stringent conditions. For analysis of TERT expression, semi
quantitative reverse transcriptase–PCR (RT-PCR) was performed
using total RNA (0.1 µg) using the Superscript Preamplification
System (GIBCO). The thermal cycles were 29 cycles of 94°C for 60
seconds, 52°C for 60 seconds, and 72°C for 90 seconds (TERT), and
25 cycles of 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for
90 seconds (GAPDH). Sense and antisense primers were as follows:
5′-GGCTCTTCTTCTACCGTAAG-3′ and 5′-TGATGCTGGT-G
ACCTCCTTCT-3′ for TERT, and 5′-ACCATGGTCCATGC
CATC-3′ and 5′-TCCACCCCTGGTGTGA-3′ for
GAPDH. Different amounts of RNA (from 0.005 to 0.25 µg) were
first used to determine the linear dose dependency and different cycle
number performed to determine linear cycle dependency.

Western Blot Analysis
Anti-rat TEP1 antibody was the kind gift of Dr F. Ishikawa.
Whole-cell lysates or fractionated samples were resolved on 4%
SDS-PAGE for TEP1 or 6% SDS-PAGE for TERT. Proteins were
transferred onto a polyvinylidene difluoride membrane (Millipore)
and incubated with anti-rat TEP1 antibody, anti-TERT antibody
(Calbiochem), or anti-FLAG antibody M2 followed by an anti-rabbit
IgG horseradish peroxidase antibody (Amersham) or anti-mouse IgG
horseradish peroxidase antibody (Jackson Immunoresearch). Spe-
cific proteins were detected using enhanced chemiluminescence
(Amersham).

Metabolic Labeling and Orthophosphate Labeling
For metabolic labeling, cultures were preincubated in labeling
medium (3 mg/L methionine and 6.3 mg/L cysteine with 5%
dialyzed FCS) for 60 minutes in the presence or absence of H7. Then
cells were incubated in the labeling medium containing 0.1 mCi/mL
[35S]methionine and cysteine (NEN) for 18 hours in the presence or
absence of H7. After incubation, cytoplasmic and nuclear extracts
were prepared and subjected to immunoprecipitation with anti
FLAG antibody. For orthophosphate labeling, cultures were prein-
cubated in labeling medium (phosphate-free) for 60 minutes. Then
cells were incubated in the labeling medium containing 0.5 mCi/mL
[32P]orthophosphate for 6 hours. After incubation, whole-cell ex-
tracts were prepared and subjected to immunoprecipitation with anti
FLAG antibody.

Results
Association of Telomerase Activity with
VSMC Growth
To investigate the association of telomerase activity with cell
proliferation, we examined telomerase activity during prolif-
eration in primary cultures of rat VSMCs. Confluent cells 7
days after serum deprivation served as day 0 sample and were
subcultured in the presence of 10% serum. Proliferating cells
were harvested every 24 hours (days 1 through 7), and
cytoplasmic extracts (4×10^5 cell equivalents) were prepared
and analyzed by stretch PCR assay. Telomerase activity
assayed by the stretch PCR method in subconfluent VSMCs
was proportional to cell number used in the range from 4×10^3
to 4×10^4. We therefore used 4×10^3 cell equivalents (10 to 15
µg) to quantify telomerase activity. Surprisingly, cytoplasmic
telomerase activity was increased, with a peak at day 2
(50-fold), but significantly decreased on days 3 to 7 at a time
of active cell proliferation (Figure 1A). Similar changes were
observed in cytoplasmic telomerase activity using human
VSMCs and A7r5 cells (data not shown). It is noteworthy that
the induction of cytoplasmic telomerase activity appeared to
precede cell proliferation. These data suggest that telomerase
activity may be involved in the modulation of cell prolifera-
tion in VSMCs.

We next examined telomerase activity in nuclear extracts
(4×10^5 cell equivalents) from the same samples in this
model. In contrast to cytoplasmic telomerase activity, nuclear
telomerase activity was gradually increased with cell prolif-
eration and reached levels 25-fold above baseline at day 6
(Figure 1B). Because 4×10^5 cell equivalents of cytoplasmic
extracts exhibited almost the same telomerase activity as
4×10^4 cell equivalents of nuclear extracts on day 0 (data not
shown), the ratio of relative cytoplasmic telomerase activity
to relative nuclear telomerase activity shown in Figure 1C
depicts the distribution of telomerase activity at each time
point within a cell. The same results were obtained using the
telomeric repeat amplification protocol (data not shown).
Thus, we conclude that most telomerase activity in the early
phase of cell proliferation is localized in the cytoplasm and,
during the log phase of cell growth, it accumulates within the
nucleus.

Expression of Telomerase Components
To investigate the potential regulatory mechanism(s) for
telomerase activity, we examined levels of the various telom-
erase components in our model. Northern blot analysis
showed that levels of the telomerase RNA component did not
change with cell growth, which indicates that VSMCs express
this component even during cell cycle arrest (Figure 2A).
Minamino and Kourembanas
Telomerase Activation by Protein Phosphorylation

Figure 1. Association of telomerase activity with cell proliferation in VSMCs. A, Cytoplasmic telomerase activity during cell proliferation in primary rat VSMCs. Confluent cells, serum-deprived for 7 days (0.05% FCS), were seeded at a density of 1.25×10⁵ cells per mm² in the presence of 10% FCS. Proliferation was determined at each time point using Trypan blue exclusion, and cell number was harvested and counted at each time point (days 1 to 7) as indicated, and 4×10⁶ cell equivalents of cytoplasmic extracts (10 to 15 μg) were analyzed for telomerase activity with stretch PCR assay. Detection of telomerase activity was performed using a 2-step process, as follows: (1) telomerase-mediated extension of forward primer and (2) PCR amplification of resultant products with forward primer and reverse primer followed by electrophoresis of the PCR-amplified telomerase products on 7 mol/L urea and 7% polyacrylamide gel. Telomerase activity was visualized by the characteristic 6-bp ladder and is represented as intensity of the entire ladder. N indicates telomerase activity in RNase-pretreated cell extracts at day 2. B, Nuclear telomerase activity during cell proliferation in rat VSMCs. 4×10⁶ cell equivalents of nuclear extracts (1 to 1.5 μg) derived from the same cell populations as in panel A were analyzed for telomerase activity with stretch PCR assay. N indicates telomerase activity in RNase-pretreated cell extracts of cells at day 6. C, Time course of relative cytoplasmic and nuclear telomerase activity in RNase-pretreated cell extracts of cells at day 6. C, Time course of relative cytoplasmic and nuclear telomerase activity compared with cell number. Values are relative to telomerase activity at day 0. Viable cell number was determined at each time point using Trypan blue exclusion, and cell number per 100 mm²-diameter plate is indicated (n=6). Data are mean±SE; n=6 for cytoplasmic and n=3 for nuclear telomerase activity.

TEP1 is known to be modified from p240 to p230, and the modification of TEP1 is implicated in the regulation of telomerase activity.⁷ We therefore performed Western blot analysis using anti-rat TEP1 antibody. As shown in Figure 2B, total protein levels of TEP1 did not change, nor did the ratio of p230 to p240 protein levels. This suggests that TEP1 expression does not contribute to the regulation of telomerase activity in VSMCs. In contrast, TERT mRNA levels were induced with cell proliferation and reached 5-fold above baseline at day 7 (Figure 2C). Western blot analysis for TERT at days 2 and 6 demonstrated that expression of TERT was increased with cell proliferation in nuclear extracts 3- to 5-fold above day 0 and also slightly increased in cytoplasmic extracts on day 2 (Figure 2D and Figure 1 in online data supplement available at http://www.circresaha.org). These modestly increased levels of TERT mRNA and protein were associated with increased telomerase activity (Figure 2D). Taken together with the time course of telomerase activity in the cytoplasm and nucleus, these protein data indicate that accumulation of TERT in the nucleus may be partially responsible for the induction of nuclear telomerase activity.

Effects of Protein Kinase Inhibitors on Cytoplasmic Telomerase Activity

Recent reports have implicated sensitivity of telomerase activity to protein phosphorylation.¹⁷ Because increased expression of the telomerase components in VSMCs could not account for the observed dramatic increase in cytoplasmic
Figure 3. Effects of protein kinase inhibitors on cytoplasmic telomerase activity. Confluent cells deprived of serum for 7 days (day 0, lane 2) were subcultured in the presence of 10% serum (day 2, lane 3) plus protein kinase inhibitors as follows (values are in μmol/L): lane 4, herbimycin A (Her, 2); lane 5, tyrphostin A25 (Tyr, 100); lane 6, H7 (50); lane 7, PKA inhibitory peptide (1); lane 8, PKC inhibitory peptide (40); lane 9, PKG inhibitory peptide (100); and lane 10, PD98059 (50). After 48 hours, cells were harvested, and cytoplasmic extracts (10 μg) analyzed for telomerase activity with stretch PCR assay. RNase-pretreated extracts of cells at day 2 were used as negative control (N; lane 1). Results shown are representative of 3 similar experiments. Relative telomerase activity values compared with day 2 (100%) are as follows: herbimycin A, 88.2 ± 3.2; tyrphostin, 112.2 ± 25.6; H7, 20.8 ± 5.6; PKA, 53.0 ± 5.1; PKC, 25.4 ± 10.3; PKG, 107.4 ± 22.8; and PD98059, 96.6 ± 6.7.

telomerase activity, we hypothesized that the activation of telomerase in the cytoplasm may be mediated by protein phosphorylation. To test our hypothesis, we examined the effects of kinase inhibitors on the activation of cytoplasmic telomerase in culture. We prepared cytoplasmic extracts of the cells at day 2 after treatment with kinase inhibitors for 48 hours and tested for telomerase activity with stretch PCR assay. As shown in Figure 3, H7 (lane 6) and PKC inhibitory peptide (lane 8), and to a lesser degree at the doses tested, PKA inhibitory peptide (lane 7), but not the tyrosine kinase inhibitors herbimycin A (lane 4) and tyrphostin (lane 5), the PKG inhibitory peptide (lane 9), or the mitogen-activated protein kinase kinase (MEK) inhibitor, PD98059 (lane 10), reduced the activation of telomerase activity. These data suggest that telomerase is activated in the cytoplasm in part by protein phosphorylation that is mediated by an H7-sensitive kinase or kinases.

Effects of Protein Kinase Inhibitors on Nuclear Telomerase Activity

To determine whether protein phosphorylation promotes the accumulation of telomerase activity in the nucleus, we examined the effects of H7 on nuclear telomerase activity. Confluent cells deprived of serum for 7 days were subcultured in the presence of 10% serum with H7. After 6 days, cells were harvested and nuclear extracts analyzed for expression of TERT and TEP1 and telomerase activity with Western blotting (top panel). The same extracts (1 μg) were examined for telomerase activity with stretch PCR assay in the presence (+) or absence (−) of RNase (bottom panel). Results shown are representative of 3 experiments. A, Inhibitory effects of H7 on nuclear telomerase activity. Confluent cells deprived of serum for 7 days were subcultured in the presence of 10% serum with H7 at indicated concentrations. After 6 days, cells were harvested, and nuclear extracts (15 μg) were prepared and analyzed for TEP1 and TERT expression by Western blotting (top panel). The same extracts (1 μg) were examined for telomerase activity with stretch PCR assay in the presence (+) or absence (−) of RNase (bottom panel). Results shown are representative of 3 experiments. B, Effects of ATP on nuclear and cytoplasmic telomerase in vitro. Confluent cells deprived of serum for 7 days were subcultured in the presence of 10% serum with or without H7 (50 μmol/L). H7 was added every 3 days. After 6 days, cells were harvested and extracts prepared. Nuclear extracts (N) (1 μg) and cytoplasmic extracts (C) (10 μg) in kinase buffer (50 mmol/L HEPES, pH 7.4, 10 mmol/L MgCl2) were then incubated in the presence (+) or absence (−) of ATP (10 mmol/L) at 30°C for 10 minutes and subjected to stretch PCR assay. C, Distribution of TERT-FLAG protein during cell growth. TERT-FLAG cells were deprived of serum for 3 days and subcultured in the presence of serum for 6 days with 10 μmol/L H7 or without H7 (D6). Whole-cell lysates (Whole, 80 μg), nuclear extracts (Nuc, 20 μg), and cytoplasmic extracts (Cyto, 100 μg) were prepared, and TERT-FLAG protein was detected by Western blot analysis using anti-FLAG antibody. D, Metabolic labeling of TERT-FLAG cells. After serum deprivation for 3 days, TERT-FLAG cells were subcultured in medium containing 10% serum. At day 4, cells were preincubated in labeling medium with or without H7 (10 μmol/L) for 60 minutes followed by incubation in labeling medium containing [35S]methionine and cysteine in the presence (H7, lanes 5 and 6) or absence of H7 (lanes 1 through 4) for 18 hours. Cytoplasmic (C) and nuclear (N) extracts from the same cell equivalents (~0.5 × 106) were immunoprecipitated with anti-FLAG antibody in the presence (+, lanes 2 and 4) or absence (−, lanes 1, 3, 5, and 6) of FLAG peptide (20 μg). Arrow indicates position of TERT-FLAG; *, unrelated bottom bands cross-reacting with antibody.

did not affect TEP1 levels in the nucleus. H7 treatment did not affect TERT levels in whole-cell extracts but increased cytoplasmic TERT with a corresponding decrease in nuclear levels of TERT (Figure 2 in online data supplement available at http://www.circresaha.org).
To obtain further evidence that protein phosphorylation is involved in the regulation of telomerase activity, we examined the effects of ATP on telomerase activity in vitro. We prepared nuclear and cytoplasmic extracts derived from cultures treated with H7 for 6 days, incubated the extracts with ATP in the presence of 10 mmol/L MgCl₂, and assayed for telomerase activity with stretch PCR. Elevated nuclear telomerase activity was observed even in the absence of exogenous ATP at basal conditions (Figure 4B, lanes 1 and 2). However, when cells were treated with H7, no nuclear telomerase activity was detectable even in the presence of exogenous ATP (lanes 3 and 4). This inability to restore nuclear telomerase activity in vitro is not due to absence of protein kinase or kinases from the nucleus. Indeed, we found that treatment of day 6 nuclear extracts with alkaline phosphatase reduced telomerase activity but the addition of ATP restored it (data not shown). This indicates that most nuclear telomerase may be in the phosphorylated form, and reduced nuclear telomerase activity as a result of treatment with H7 could represent decreased levels of telomerase in the nucleus consistent with Western blotting (Figure 4A). In contrast, ATP enhanced telomerase activity in cytoplasmic extracts derived from cultures treated with H7 (lanes 5 and 6), which suggests that the majority of unphosphorylated telomerase remains in the cytoplasm.

To confirm the effects of H7 on the accumulation of TERT in the nucleus, we established VSMC lines that constitutively express TERT-FLAG protein and examined the distribution of TERT-FLAG during cell growth. TERT-FLAG—expressing cells deprived of serum for 3 days were subcultured with the addition of serum for 6 days with or without H7, and TERT-FLAG protein levels were assessed by Western blotting in whole-cell, nuclear, and cytoplasmic extracts (Figure 4C). TERT protein remained constant in whole-cell extracts for the entire 6-day culture period even in the presence of H7. However, TERT-FLAG was absent in nuclear extracts on day 0 (D0) and appeared on day 6 (D6), correlating with high nuclear telomerase activity (Figures 1C and 2D). Conversely, TERT-FLAG protein was immunoprecipitated with anti-FLAG antibody. In addition to TERT-FLAG, multiple 35 S-labeled bands (*) appeared in the nuclear extracts but were unrelated to TERT-FLAG, as shown by the inability to be competed by FLAG peptide. Consistent with the distribution of telomerase activity at days 4 to 5 (Figure 1C), almost equal amounts of TERT-FLAG protein were detected in the cytoplasm and nucleus (Figure 4D, lanes 1 and 3). In contrast, TERT-FLAG protein remained in the cytoplasm in cells treated with H7 (lanes 5 and 6). It is noted that a <10% reduction in labeling rates was seen in the extracts of H7-treated cells, compared with the control cells. Taken together, these data indicate that protein phosphorylation is required for the accumulation of TERT as well as telomerase activity in the nucleus. Moreover, it appears that phosphorylation of TERT may be critical for TERT function and regulation of VSMC proliferation. Treatment of cells with H7 (50 μmol/L) significantly inhibited VSMC proliferation (data not shown).

**Phosphorylation of TERT**

Because TEP1 protein levels in the nucleus were unaffected by treatment with H7, we speculated that protein phosphorylation of TERT might be responsible for activation and accumulation of telomerase in the nucleus. Cytoplasmic extracts from TERT-FLAG cells deprived of serum for 3 days were subjected to in vitro kinase reaction in the presence of 1 mmol/L ATP and 1 μCi/μL [γ-32P]ATP for 0 (lane 1) or 10 (lane 2) minutes with 10 μmol/L H7 (lane 3) or 40 μmol/L PKC inhibitory peptide (lane 4). In vitro kinase reaction mixtures were then immunoprecipitated with anti-FLAG antibody. One tenth of each immunoprecipitate was assayed for telomerase activity (bottom panel). B. Protein phosphorylation of TERT-FLAG in VSMCs. VSMCs transfected with TERT-FLAG expression vector were labeled with [32P]orthophosphate for 6 hours with (lane 2) or without H7 (lanes 1 and 3). TERT-FLAG protein was immunoprecipitated with anti-FLAG antibody in the absence (lanes 1 and 2) or presence of FLAG peptide (lane 3). Immunoprecipitates were subjected to SDS-PAGE (6%) analysis and transferred onto a polyvinylidene difluoride membrane followed by autoradiography to detect phosphorylated TERT-FLAG (p–TERT-FLAG) and immunoblotting with anti-FLAG antibody to detect immunoreactive TERT-FLAG (ir–TERT-FLAG).
Telomerase Inhibition Reduces VSMC Proliferation

To investigate whether telomerase activation contributes to VSMC growth, we inhibited telomerase activity using a putative telomerase inhibitor (TAG). TAG comprises hexameric, telomere-mimicking phosphorothioate oligonucleotides, TTAGGG, that have been shown to suppress telomerase activity in cancer cells. VSMCs deprived of serum for 7 days were stimulated to proliferate by addition of serum in the presence of TAG or scrambled phosphorothioate oligonucleotides (Scramble, 5 μmol/L) or scrambled phosphorothioate oligonucleotides (Scramble, 5 μmol/L). Two days after incubation, cultures were harvested and cytoplasmic extracts (10 μg) analyzed for telomerase activity with stretch PCR assay. Day 2 sample pretreated with RNase served as negative control (N). B, Telomerase inhibition diminishes cell growth. VSMCs deprived of serum for 7 days were stimulated to proliferate in the absence (Control) or presence of telomerase inhibitor (TAG) or scrambled phosphorothioate oligonucleotides (Scramble). Four days after incubation, cultures were harvested and cell number was determined using Trypan blue exclusion. Values are relative cell number to control at day 4. Data are mean±SE (n=4). TAG significantly inhibited VSMC proliferation in a dose-dependent manner (P<0.0005, **P<0.0001 vs Control, one-way ANOVA).

Discussion

Proliferation of VSMCs underlies the pathophysiology of vascular diseases, such as atherosclerosis and restenosis after vascular injury.18 We recently reported that hypoxia, a well-known regulator of vascular tone and wall structure, extends the life span of VSMCs through telomerase activation.19 Understanding mechanisms of VSMC proliferation would provide insight into prevention and treatment of important vascular disorders. In the present study, we demonstrated that telomerase was activated during cell proliferation in VSMCs, whereas inhibition of telomerase activity resulted in decreased cell growth. These data suggest that telomerase activation is crucial in the regulation of VSMC proliferation. However, the molecular mechanisms by which inhibition of telomerase reduces proliferation in VSMCs remain unclear.

We have made the surprising observation that cytoplasmic telomerase activity was induced before cell proliferation whereas nuclear telomerase activity correlated with cell proliferation. We did not observe great changes in expression levels of telomerase components compared with the dramatic induction of its activity during cell proliferation, indicating that telomerase activity may be regulated by posttranslational modification rather than at the level of component expression. Protein phosphorylation appeared to play a key role in the regulation of telomerase activity. First, protein kinase inhibitors suppressed cytoplasmic telomerase activation in culture. Second, inhibition of protein kinases also reduced the accumulation of TERT protein and telomerase activity in the nucleus, whereas TEP1 protein levels in the nucleus were unaffected. Finally, TERT was found to be phosphorylated in culture by an H7-sensitive kinase. Interestingly, cytoplasmic telomerase activity in cells treated with H7 was recovered by ATP whereas ATP failed to restore nuclear telomerase activity in vitro. Because we and others16 showed that nuclear telomerase activity is reduced by protein phosphatase treatment but restored with ATP in vitro, our inability to restore nuclear telomerase activity in the presence of H7 was not due to the absence of protein kinase or kinases from the nucleus. Our findings indicate that most telomerase in the nucleus is phosphorylated whereas both phosphorylated and unphosphorylated forms exist in the cytoplasm. Indeed, TERT is shuttled between the cytoplasm and nucleus from day 0 to day 6, but H7 treatment prevented the detection of TERT in the nucleus and caused it to remain in the cytoplasm on day 6, a time when TERT is normally undetectable there. This is further confirmed by the pulse-chase experiments whereby H7 treatment prevented the accumulation of TERT in the nucleus but did not alter TERT levels in the cytoplasm. Thus,
it is conceivable that a protein kinase or kinases activate telomerase in the cytoplasm, and only an active form or forms of telomerase are capable of accumulating into the nucleus. Whether an identical protein kinase or kinases are responsible for both TERT phosphorylation and telomerase translocation remains to be elucidated. Telomerase phosphorylation, however, is likely to be critical for VSMC proliferation because treatment with H7 inhibited nuclear telomerase activity (Figure 4B) as well as cell proliferation (data not shown). In our model, a significant reduction in cell proliferation was observed after treatment with telomerase inhibitors (Figure 6B), further confirming the critical role of telomerase in regulating VSMC growth. Because this occurred within 4 days of telomerase inhibition, it is unlikely that progressive telomere shortening was responsible for decreased cell proliferation. Rather, inhibition of telomerase may promote cell cycle arrest in VSMCs, as has been reported for cancer cells.20 In the latter case, increased cyclin-dependent kinase (CDK) inhibitors have been observed. Because we also observed increased CDK inhibitors in VSMCs treated with the telomerase inhibitor (our unpublished observations, 2000), reduced cell proliferation induced by telomerase inhibition in VSMCs may be attributed to induction of CDK inhibitors.

In summary, we have demonstrated that telomerase is first activated in the cytoplasm before cell proliferation, followed by accumulation of activity in the nucleus. We speculate that protein phosphorylation of TERT is responsible for activation of telomerase in the cytoplasm, which in turn leads to the accumulation of telomerase into the nucleus, as occurs with certain transcription factors. Consistent with this, we noticed that human TERT contains a classical nuclear localization signal (amino acids 223 to 240), thus implicating an importin-dependent translocation mechanism for TERT.21 Moreover, we observed that telomerase activity was associated with TERT phosphorylation. Our data on telomerase inhibition suggest telomerase as a potentially important target for the treatment of vascular diseases. Thus, additional studies on the regulation of telomerase activity are critical for further understanding the role of telomerase in the vasculature and for developing novel therapeutic strategies in human vascular disorders.

Acknowledgments

This work was supported by the American Heart Association and by NIH Grants RO1 HL55454 and SCOR 1P50 HL56398. We thank F. Ishikawa for pcDNA3 TERT-FLAG and anti-rTEP1 antibodies and S.A. Mitsialis for helpful suggestions. We also thank Jessica Johnson for her expert assistance in the preparation of this manuscript.

References

Mechanisms of Telomerase Induction During Vascular Smooth Muscle Cell Proliferation
T. Minamino and S. Kourembanas

Circ Res. published online July 19, 2001;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2001/07/19/hh1501.094267

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2001/07/20/hh1501.094267.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplement

Figure 1

Western blot analysis for TERT expression. Cytoplasmic (100 μg) and nuclear extracts (20 μg) were analyzed for TERT expression using anti-TERT antibody at Day 0, 2 and 6. To assess sample purity, the same fractionated samples were examined for Sp1 or beta actin (lower panel). Similar results were observed in three independent experiments.

Figure 2

Inhibitory effects of H7 on TERT subcellular localization. Confluent cells deprived of serum for 7 days were subcultured in the presence of 10% serum with H7 at the indicated concentrations. After 6 days, cells were harvested, nuclear extracts (Nuc, 15 μg), whole cell extracts (Whole, 100 μg) and cytoplasmic extracts (Cyto, 100 μg) were prepared and analyzed for TERT content by Western blotting.
Supplement

**Fig 1**

<table>
<thead>
<tr>
<th>Day</th>
<th>Nuc</th>
<th>Cyto</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

TERT

Sp1

Actin

**Fig 2**

H7 0 15 50 μmol/L

TERT (nu)

TERT (cyto)

TERT (whole)