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.
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 (Oncof) was also used for telomerase activity assay and performed according to the manufacturer’s instructions.16 To confirm the linearity of the strech PCR assay, we performed pilot experiments for each extract. According to these experiments, 4 × 10^4 cell equivalents were subjected to strech 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 with 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′-GGCTTCTTCTTACCGTAAG-3′ and 5′-TGATGGGCTTG-ACCTTCTTCTTG-3′ for TERT, and 5′-ACCUGAGATTTCATGACGACCATCAC-3′ and 5′-TCCACCCACGTGTTGCTGA-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). Specific 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 preincubated 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 extracts 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 proliferation 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 × 10^4 cell equivalents) were prepared and analyzed by strech PCR assay. Telomerase activity assayed by the strech PCR method in subconfluent VSMCs was proportional to cell number used in the range from 4 × 10^4 to 4 × 10^6. We therefore used 4 × 10^5 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 proliferation in VSMCs.

We next examined telomerase activity in nuclear extracts (4 × 10^4 cell equivalents) from the same samples in this model. In contrast to cytoplasmic telomerase activity, nuclear telomerase activity was gradually increased with cell proliferation and reached levels 25-fold above baseline at day 6 (Figure 1B). Because 4 × 10^4 cell equivalents of cytoplasmic extracts exhibited almost the same telomerase activity as 4 × 10^5 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 telomerase 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).
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 1.25×10^5 cells per mm^2 in the presence of 10% FCS. Proliferation was 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. 4×10^4 cell equivalents of nuclear extracts (1 to 1.5 μg) were analyzed for telomerase activity with stretch PCR assay. N indicates conditions under which 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. 4×10^4 cell equivalents of nuclear extracts (1 to 1.5 μg) were analyzed for telomerase activity with stretch PCR assay. N indicates telomerase activity in RNase-pretreated cell extracts at day 2. C, Time course of relative cytoplasmic and nuclear telomerase activity compared with day 0 from 3 independent experiments are as follows: Nuclear, 2.68±0.9 (day 2) and 4.8±2 (day 6), and cytoplasmic, 1.8±0.25 (day 2) and 1.12±0.06 (day 6). The same fractionated samples (1 μg for nuclear fractions and 10 μg for cytoplasmic fractions) were examined for telomerase activity (bottom panel). Similar results were observed in 3 independent experiments.

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Figure 2. Expression of telomerase components in rat VSMCs. A, Northern blot analysis for the telomerase RNA component. Total RNA (30 μg) was probed for telomerase RNA component expression (top panel) at indicated time points after subculture on day 0 in the presence of 10% serum. Ribosomal 28S was used for internal control (bottom panel). B, Western blot analysis for TEP1 expression. Whole-cell lysates (30 μg) were analyzed for TEP1 expression at the same time points as in panel A using anti-rat TEP1 antibody. Positions of p240 and p230 are indicated. Equality of loading samples was confirmed by Ponceau S staining. C, TERT expression. Total RNA samples (0.1 μg) were assayed for expression of TERT (top panel) and GAPDH (bottom panel) at the same time points as in panel A by RT-PCR. Right panel indicates a standard reaction using different amounts of total RNA from day 7 samples (0.005, 0.025, and 0.25 μg) to determine linear dose dependency under these conditions. D, Induction of TERT protein and telomerase activity. Nuclear (20 μg; Nuc) and cytoplasmic (100 μg; Cyto) fractions were assayed for TERT protein expression by Western blotting (top panel). Mean relative values of TERT protein induction on days 2 and 6 compared with day 0 from 3 independent experiments are as follows: Nuclear, 2.68±0.9 (day 2) and 4.8±2 (day 6), and cytoplasmic, 1.8±0.25 (day 2) and 1.12±0.06 (day 6). The same fractionated samples (1 μg for nuclear fractions and 10 μg for cytoplasmic fractions) were examined for telomerase activity (bottom panel). Similar results were observed in 3 independent experiments.

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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...
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 at indicated concentrations. After 6 days, cells were harvested, and nuclear extracts (10 μg) were prepared and analyzed for TERT expression 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. 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) (100 μg) were incubated in labeling medium with 50 mmol/L HEPES, pH 7.4, 10 mmol/L MgCl2, ATP (10 mmol/L) at 30°C for 10 minutes and subjected to stretch PCR assay. C, Distillation 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, it was detected in cytoplasmic extracts on day 0 but was absent on day 6. Treatment of cells with the kinase inhibitor H7 prevented the detection of TERT-FLAG in the nucleus on day 6 (Figure 4C, lane 6) but allowed it to accumulate and be detected in the cytoplasm (lane 9). Metabolic labeling with [³²S]methionine and cysteine in the presence or absence of H7 was performed to follow the distribution of TERT-FLAG between the cytoplasmic and nuclear components. After serum deprivation for 3 days, TERT-FLAG cells were grown in medium containing 10% serum. At day 4, when TERT-FLAG protein would be actively accumulating into the nucleus, cells were labeled with [³²S]methionine and cysteine in the presence or absence of H7 for 18 hours. Cytoplasmic and nuclear extracts from the same cell equivalents (≈0.5×10⁶) were then immunoprecipitated with anti-FLAG antibody. In addition to TERT-FLAG, multiple [³²S]-labeled bands (*) appeared in the nuclear extracts but were unrelated to TERT-FLAG, as shown by the inability to be competed with 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 [³²P]ATP plus H7 or PKC inhibitory peptide followed by immunoprecipitation with anti-FLAG antibody. Although TERT-FLAG was phosphorylated in control extracts (Figure 5A, lane 2) and resulted in increased telomerase activity (bottom panel), the presence of H7 or PKC inhibitory peptide inhibited this phosphorylation and corresponding telomerase activity (Figure 5A, lanes 3 and 4). To determine whether
Figure 6. Effects of telomerase inhibition on VSMC proliferation. A, Inhibitory effects of TAG on telomerase activity. VSMCs deprived of serum for 7 days (day 0) were stimulated to proliferate by addition of 10% serum in the absence (day 2) or the presence of 1 (TAG 1) or 5 (TAG 5) μmol/L telomerase inhibitor, 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).

TERT is phosphorylated in VSMCs, TERT-FLAG cells were labeled with [32P]orthophosphate for 6 hours and TERT-FLAG protein was immunoprecipitated with anti-FLAG antibody. As expected, phosphorylated TERT-FLAG was detected in VSMCs, whereas preincubation with FLAG peptide completely abolished detection of phosphorylated TERT-FLAG as well as immunoreactive TERT-FLAG (Figure 5B). Treatment with H7 inhibited phosphorylated TERT-FLAG detection, but immunoreactive TERT-FLAG was unaffected (Figure 5B, lane 2). Thus, protein phosphorylation of TERT may be an important mechanism underlying the regulation of telomerase activity in VSMCs.

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, TGTGAG, and cell number and telomerase activity were determined. As shown in Figure 6A, whereas scrambled oligonucleotides did not affect telomerase activity, TAG effectively inhibited the activity in a dose-dependent manner 2 days after treatment. It is noteworthy that no significant reduction in VSMC growth was observed in cultures treated with either TAG or scrambled oligonucleotides at this time point. However, TAG significantly inhibited VSMC proliferation in a dose-dependent manner as compared with control cultures 4 days after treatment (Figure 6B). No remarkable cell death was noted in all cultures (data not shown). Consequently, these findings combined with the inhibition of cell growth on H7 treatment suggest an important role for telomerase activation in the control of VSMC proliferation.

Discussion
Proliferation of VSMCs underlies the pathophysiology of vascular diseases, such as atherosclerosis and restenosis after vascular injury. We recently reported that hypoxia, a well-known regulator of vascular tone and wall structure, extends the life span of VSMCs through telomerase activation. 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 others 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. 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. 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.

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References


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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.