Fibroblast Growth Factor-2, But Not Vascular Endothelial Growth Factor, Upregulates Telomerase Activity in Human Endothelial Cells

David J. Kurz, Ying Hong, Elizabeth Trivier, Hsiu-Lin Huang, Stephanie Decary, Guo Hong Zang, Thomas F. Lüscher, Jorge D. Erusalimsky

Objective—Telomerase plays a major role in the control of replicative capacity, a critical property for successful angiogenesis and maintenance of endothelial integrity. In this study, we examined the relationship between telomerase activity and endothelial cell proliferation as well as the regulation of this enzyme by fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor-A (VEGF).

Methods and Results—Telomerase was repressed in endothelial cells freshly derived from intact endothelium, whereas activity was present during logarithmic growth in culture. In cultured human umbilical vein endothelial cells (HUVECs), mRNA levels of hTER—the catalytic subunit of telomerase—and enzyme activity decreased reversibly on induction of quiescence. Treatment of quiescent HUVECs with FGF-2 restored telomerase activity in a time- and dose-dependent manner, whereas VEGF had no such effect, although both factors induced comparable mitogenic responses. FGF-2, but not VEGF, upregulated the mRNA levels for hTERT and for the hTERT gene transactivation factor Sp1. Serial passage in the presence of individual growth factors accelerated the accumulation of senescent cells in VEGF-treated cultures compared with cultures treated with FGF-2.

Conclusions—FGF-2, but not VEGF, restores telomerase activity and maintains the replicative capacity of endothelial cells. (Arterioscler Thromb Vasc Biol. 2003;23:●●●●●●●.)

Key Words: telomerase • fibroblast growth factor-2 • endothelial cell • Sp1 • senescence

Induction of angiogenesis is a novel therapeutic strategy presently being evaluated for the treatment of human ischemic vascular disease. The success of this strategy depends on an adequate proliferative response of vascular endothelial cells at the site of ischemia.1 Cellular replicative potential is in part limited by the integrity of telomeres, the physical ends of chromosomal DNA.2 Telomeric DNA shortens by ≈50 to 200 base pairs with each round of cell division as a consequence of the inability of conventional DNA polymerases to replicate the 3' termini of the template strands. A large body of evidence indicates that critical shortening of one or more telomeres activates a DNA damage checkpoint that blocks additional replication and leads to cell senescence. However, more recently, the integrity of the T-loop—a higher-order structure formed at the end of the telomere by telomeric DNA and specialized proteins—rather than telomere length, per se, has been implicated as a major determinant of senescence.3,4

In many actively proliferating cells, maintenance of replicative capacity and chromosome stability requires the activity of telomerase, a specialized reverse-transcriptase that adds hexanucleotide repeats of the sequence TTAGGG to the 3' ends of nuclear DNA, thus counteracting telomeric erosion.3 Studies in various immortalized cell lines5 and normal human lymphocytes6 have shown that like other enzymes involved in chromosomal DNA synthesis,7 telomerase activity levels are differentially regulated during periods of proliferation and quiescence. Human telomerase consists of at least 3 subunits. Of these, the RNA component hTR, which provides the template for telomere synthesis, and the telomerase-associated protein hTEP1 are constitutively expressed.8,9 The third component, human telomerase reverse transcriptase (hTERT), which contains the catalytic activity of telomerase, is tightly regulated in its expression4 and, thus, in most cases is responsible for the variations in telomerase activity levels.

In human postnatal life, besides its pathological upregulation in many tumor cells,10 telomerase activity can be detected in the germ line and in some normal somatic tissues that undergo sustained proliferative renewal, including hematopoietic lineages, the epidermis, and the intestine.6 The
presence of telomerase activity in cultured endothelial cells has been previously reported,\textsuperscript{11} and both NO and posttranscriptional modification by the serine/threonine kinase Akt have been implicated in the regulation of its activity.\textsuperscript{12,13} Nonetheless, the presence of telomerase activity in the endothelium in situ has not been evaluated. Furthermore, the extent to which telomerase activity is modulated by proliferation and the role of individual growth factors in its regulation in endothelial cells have not been thoroughly investigated.

In this study, we demonstrate that telomerase activity is repressed in cells freshly isolated from normal vascular endothelium but that it is reversibly upregulated according to the proliferative status. We additionally examined the impact of individual mitogens on telomerase restoration in endothelial cells, focusing on fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor A (VEGF). Both of these mitogens profoundly enhance endothelial regrowth in vivo and have potent angiogenic properties.\textsuperscript{14–16} We show that telomerase activity and the mRNA levels of both hTERT and the hTERT gene transactivation factor Sp1 are restored when growth is stimulated by FGF-2 but not by VEGF. Furthermore, we demonstrate an increased accumulation of senescent cells in cultures grown in the presence of VEGF compared with cultures grown with FGF-2.

### Methods

#### Isolation of Endothelial Cells

Umbilical cords were obtained from term deliveries by cesarean section. Porcine aortae were from a local abattoir. Endothelial cells were harvested within 3 hours by collagenase digestion, as previously described.\textsuperscript{17} Detached endothelial cells were washed in PBS and processed directly for measurement of telomerase activity or resuspended in endothelial cell growth medium-2 (EGM-2) (BioWhittaker) for cell culture.

#### Cell Culture

Freshly isolated or commercially available (Biowhittaker) endothelial cells were grown in EGM-2, consisting of modified CCMD130 medium supplemented with 2\% FCS, hydrocortisone, human FGF-2, VEGF, R\textsuperscript{3}-insulin-like growth factor-1, human epidermal growth factor, heparin, ascorbic acid, gentamycin, and amphotericin B, as supplied by the manufacturer. Cells were cultured in 25-cm\textsuperscript{2} flasks (Falcon) or 6-well plates, as previously described.\textsuperscript{18} Unless otherwise indicated, experiments were performed on cells that had previously undergone 2 passages (ie, 6 to 7 population doublings). To induce quiescence of human umbilical vein endothelial cells (HUVECs) by growth factor withdrawal, cultures were transferred 2 days after plating to a basal medium containing ascorbic acid, heparin, and 1\% FCS but lacking any other growth factor supplements and maintained under these conditions for 3 days, unless otherwise indicated. To induce replicative senescence, cells were serially passaged as detailed in the online data supplement (see http://atvb.ahajournals.org). Human recombinant FGF-2, VEGF, goat anti-human FGF-2 neutralizing antibodies, and goat IgG isotype control antibodies were from R&D Systems.

#### Telomerase Assay

Endothelial cells were harvested by trypsinization and lysed at 4°C in 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid buffer. Aliquots of the cleared lysate equivalent to 2×10\textsuperscript{5} cells (or 1.5 μg of protein, where indicated) were assayed for telomerase activity by a modified telomeric repeat amplification protocol (TRAPeze, Intergen), which is based on the method originally described by Kim et al.\textsuperscript{10} Telomerase activity was calculated from the ratio of the intensity of the telomeric repeat ladder (starting at 50 bp) to the 36-bp internal standard. Results, expressed in arbitrary units (AU), were normalized to the signal obtained from an extract of 500 HeLa cells routinely assayed in parallel.

#### Reverse Transcriptase–Polymerase Chain Reaction

Analysis of RNA

Total RNA was extracted from cell monolayers using TRIzol (Invitrogen). Synthesis of cDNA templates and polymerase chain reaction (PCR) were carried out as detailed in the supplementary online information (see http://atvb.ahajournals.org). Amplification products were visualized by ethidium bromide staining under UV illumination and photographed using a GeneGenius gel documentation system (Syngene, Synoptics).

#### Telomere Length Analysis

Genomic DNA was extracted at the end of each passage and digested with Hinfl/RsaI. Southern blot analysis of terminal restriction fragments (TRF) was carried out by hybridization to a telomere-specific probe followed by chemiluminescence detection (Telomerase detection system, Roche Molecular Biochemicals).

#### Senescence Associated β-Galactosidase Assay

Cytochemical staining for senescence associated β-galactosidase (SA-β-gal) was performed at pH 6, as previously described.\textsuperscript{18} Stained cultures were viewed under an Axiovert 25CFL inverted microscope (Carl Zeiss) under bright field illumination at ×100 magnification, using a green conversion filter. Representative fields were photographed using Kodak Ektachrome 64T color positive film.

#### Statistical Analysis

Experiments were performed at least 3 times. Levels of statistical significance were determined by Student’s unpaired, 2-tailed t test or by ANOVA followed by Bonferroni’s multitest correction, as indicated. Results are presented as mean±SD.

#### Results

### Low Levels of Telomerase Activity in the Endothelium Are Increased by Cell Culture

The telomeric repeat amplification protocol (TRAP) is a two-stage reaction, the first of which involves the extension of a substrate oligonucleotide with TTAGGG repeats by the telomerase present in cell or tissue lysates. In the second step, these extension products are amplified by the polymerase chain reaction, generating a characteristic telomeric repeat ladder with 6-bp increments.\textsuperscript{10} Figure 1A shows that this assay can readily detect telomerase activity in cultured HUVECs and that a linear correlation exists between the number of cells assayed and the measured activity. Based on these findings, aliquots corresponding to 2×10\textsuperscript{4} cells were used in subsequent experiments. The activity in this number of cells was comparable to that of 500 HeLa cells, indicating that in HUVECs, telomerase activity is ~40-fold lower than in a typical telomerase-positive tumor cell line. Similar levels of activity were found in cultured adult human aortic and microvascular endothelial cells (data not shown).

To examine if telomerase activity was also present in the endothelium in situ, we isolated endothelial cells from human umbilical veins and from adult porcine aortas. As shown in Figure 1B, these cells had very low telomerase activity (0.62±0.06 AU in HUVECs and 0.53±0.24 AU in porcine endothelial cells). However, telomerase activity was upregu-
ulated 4- to 6-fold after these cells had been grown in culture for 3 days. Mixing experiments demonstrated that these differences in activity were not attributable to the presence of TRAP reaction inhibitors in the uncultured samples (data not shown).

Telomerase Activity in Endothelial Cells Is Growth Regulated

The above results suggested that telomerase activity in endothelial cells is upregulated during cell proliferation. To investigate this possibility in more detail, telomerase activity was measured in cultured HUVECs maintained in exponential growth or induced to undergo quiescence. Figure 2A shows that HUVECs kept in exponential growth maintain a relatively high level of telomerase activity. In contrast, HUVECs undergoing quiescence as a result of growth factor withdrawal (Figure 2B) or contact inhibition (Figure 2C) showed a slow and progressive drop in telomerase activity. Furthermore, when quiescence was imposed by the combined action of contact inhibition and growth factor withdrawal, a faster decrease in telomerase activity was observed (Figure 2D). Consistent with these findings, reverse transcriptase (RT)-PCR analysis demonstrated that after induction of quiescence levels of hTERT mRNA were markedly reduced, suggesting that downregulation occurs primarily at the transcript level (Figure 2E). The downregulation of telomerase activity could be fully reversed when nonconfluent quiescent cells were reexposed to the full complement of growth factors present in EGM-2 (Figure 2F). In contrast, if cultures were confluent, addition of growth factors caused only a minor
although significant increase in telomerase activity. These findings suggested that full upregulation of telomerase activity was possible only if the constraints imposed by contact inhibition were removed.

FGF-2, But Not VEGF, Upregulates Telomerase Activity in HUVECs

To gain additional insight into the regulation of telomerase activity in endothelial cells, the effect of individual growth factors and hormones present in EGM-2 was examined. Preliminary experiments showed that when hydrocortisone, VEGF, R3-insulin–like growth factor-1, epidermal growth factor, and FGF-2 were added individually to the basal medium, only FGF-2 upregulated telomerase activity (data not shown). To additionally substantiate the existence of this differential effect, we took advantage of the fact that VEGF and FGF-2 elicit similar mitogenic responses in endothelial cells and compared these 2 factors in more detail. As shown in Figure 3A, at 25 ng/mL, both mitogens caused a very similar proliferative response. In contrast, whereas telomerase activity was upregulated in response to FGF-2 in a time- and dose-dependent manner, it remained suppressed at all times and concentrations of VEGF tested (Figures 3B, 3C, and 3D). Maximal effect of FGF-2 was observed at a concentration of 25 ng/mL (2.31 ± 0.42 AU versus 1.0 ± 0.17 AU for basal medium; \(P<0.001\), Figure 3D), with higher concentrations (up to 100 ng/mL) causing no additional increases in activity levels (data not shown). Simultaneous stimulation with VEGF and FGF-2 had no discernible effect over that seen in the presence of FGF-2 alone (Figure 3B).

Upregulation of Telomerase Activity by FGF-2 Is Accompanied by an Increase in hTERT and Sp1 Transcripts

The relatively slow restoration of telomerase activity observed in the presence of FGF-2 (see Figure 3C) suggested that this process is governed primarily by a transcriptional mechanism. To verify this possibility, we examined the
effects of FGF-2 on the mRNA levels of hTERT and those of transcription factors that had previously been implicated in the regulation of this gene in other cell types. The hTERT core promoter region is rich in E-boxes binding c-Myc/Max and GC-boxes binding Sp1.19 Although all of these factors positively regulate transcriptional initiation of hTERT, Mad1, which dimerizes with c-Myc in competition with Max, has been shown to repress hTERT expression.20

As shown in Figure 4, hTERT mRNA levels increased after stimulation of quiescent HUVECs with FGF-2, but not when the same cells were stimulated with VEGF. It was consistently noted that hTERT mRNA levels were lower in FGF-2–stimulated cells compared with cultures growing exponentially in complete medium, in agreement with the results from the TRAP assay (see Figure 3B). FGF-2 also increased Sp1 and c-Myc mRNA levels, whereas VEGF upregulated only c-Myc. In contrast, levels of Max or Mad1 transcripts did not change significantly after stimulation with either growth factor (Figure 4).

Figure 3. FGF-2, but not VEGF, upregulates endothelial cell telomerase activity. A and B, Nonconfluent, quiescent HUVECs were incubated for 2 days with fresh basal medium (BM) or fresh BM supplemented with either 25 ng/mL VEGF, 25 ng/mL FGF-2, a combination of 25 ng/mL VEGF and FGF-2, or EGM-2, as indicated. Values represent the mean (±SD, n=8) fold increase in cell density (A) and telomerase activity (B). C, Mean telomerase activity levels (±SD, n=3) after stimulation of quiescent HUVECs for the indicated periods of time with either 25 ng/mL VEGF (○) or 25 ng/mL FGF-2 (●). D, as in B, except that cells were incubated for 2 days with increasing concentrations of FGF-2 (black bars) or VEGF (white bars), n=4. *P<0.05, **P<0.01, ***P<0.001 by ANOVA, with Bonferroni’s multiple test correction where appropriate.

Figure 4. FGF-2, but not VEGF, upregulates hTERT and Sp1 mRNA levels. Nonconfluent HUVECs were made quiescent by incubation in basal medium supplemented with 0.2% FCS for 24 hours. Cells were then treated for 6 hours without (BM) or with either 25 ng/mL VEGF or 25 ng/mL FGF-2. RT-PCR analysis for the indicated transcripts was performed as described in Methods.
Factors Other Than FGF-2 Are Also Involved in the Regulation of Telomerase Activity in Endothelial Cells

In the experiments described above, FGF-2 caused only a partial restoration of hTERT mRNA and telomerase activity. In agreement with these results, Figure 1 (see the online data supplement, available at http://atvb.ahajournals.org) shows that omission of FGF-2 from EGM-2 led to a partial decrease in telomerase activity. Furthermore, blocking the activity of residual FGF-2 bound to the extracellular matrix with FGF-2-neutralizing antibodies resulted in an additional significant decrease in telomerase activity. Nonetheless, even under these conditions, telomerase activity was still significantly higher than in cultures maintained in basal medium, suggesting that factors other than FGF-2 must also be involved in its activation.

Effects of FGF-2 and VEGF on the Replicative Lifespan of HUVECs

To evaluate the significance of telomerase upregulation by FGF-2, we investigated whether serial passage of HUVECs in the presence of individual growth factors would differentially affect telomere length and the onset of senescence. TRF analysis during subculture for 3 passages with either FGF-2 or VEGF alone revealed no significant differences in telomere length between treatments (data not shown). Additional subculture in the presence of individual growth factors was not feasible, because cell expansion was impaired. Nevertheless, there were substantial morphological differences between the 2 treatments. In the presence of FGF-2, cells retained typical cobblestone morphology and continued to replicate, although at the same time cultures had increased numbers of dead cells (data not shown). In contrast, cultures maintained in the presence of VEGF ceased to replicate, displaying an increase in cell size and numerous flat, binucleated cells, reminiscent of a senescent phenotype. Furthermore, as shown in Figure II (see the online data supplement, available at http://atvb.ahajournals.org), in VEGF-treated cultures, a large proportion of cells stained positive for SA-β-gal, an established biomarker of senescence, whereas cultures grown with FGF-2 had few SA-β-gal-positive cells. Similarly, morphological features of senescence and a delayed cell growth were observed already after 1 passage in EGM-2 lacking FGF-2 and containing 1 μg/mL of FGF-2-neutralizing antibody (2.44 versus 4.23 population doublings, respectively), with additional passage under these conditions also being impaired. Thus, despite the presence of several other growth factors, including VEGF, and in agreement with the notion that HUVECs are more fastidious in their growth requirements than other large-vessel endothelial cells, long-term culture was not possible in the absence of FGF-2.

In apparent contrast to our findings, it has previously been reported that VEGF delays the onset of replicative senescence in human dermal microvascular endothelial cells. To investigate whether the same was true for HUVECs, these were subcultured until senescence in the absence or presence of 5 ng/mL VEGF in a medium supplemented with bovine brain extract as a source of FGF-2. As shown in Figure III (see the online data supplement, available at http://atvb.ahajournals.org), supplementation with VEGF did not delay the onset of senescence, as determined either by growth characteristics (Figure IIIA) or by the increase in the percentage of SA-β-gal positive cells (Figure IIIB).

Discussion

The present study demonstrates that in normal endothelial cells, telomerase activity is growth regulated and that FGF-2 plays a major role in the restoration of this activity on exit from quiescence. These conclusions are based on the following findings. First, telomerase activity was virtually absent in cells freshly isolated from aortic or umbilical vein endothelium, where the cells are normally maintained in a quiescent state, but was markedly upregulated when the same cells were placed in culture and stimulated to proliferate (Figure 1B). Second, during exponential growth, telomerase activity was maintained at a relatively constant level (Figure 2A), whereas culture conditions leading to quiescence caused a significant downregulation of both telomerase activity (Figures 2B, 2C, and 2D) and hTERT mRNA expression (Figure 2E). Third, reestablishment of growth was associated with a complete recovery of telomerase activity (Figure 2F). Finally, among a series of individual endothelial cell mitogens, FGF-2 was the only one with the capacity to restore telomerase activity on its own (Figure 3). These findings are in agreement with studies in immortal cell lines and lymphocytes showing that telomerase activity is elevated in actively cycling cells but is downregulated in quiescent cells.6,7 They also concur with reports showing that FGF-2 upregulates telomerase activity in neural precursor cells, synoviocytes, and lung fibroblasts.8

Others have recently reported that cultured endothelial cells are telomerase competent and that activity is repressed on growth inhibition and replicative senescence. The present study extends these findings by demonstrating that telomerase activity could be restored on the reinitiation of growth. These experimental conditions may more closely represent in vivo situations, such as episodes of angiogenesis, where quiescent endothelial cells are forced to resume cell division. Furthermore, our observation that telomerase could be reversibly downregulated in culture enabled us to test the effect of individual growth factors on this activity. Indeed, a major finding of this study is the demonstration that in HUVECs, FGF-2 induced an increase of both telomerase activity (Figure 3) and hTERT mRNA expression (Figure 4). These effects were highly selective for FGF-2, because VEGF, which under the present experimental conditions had a similar mitogenic capacity (Figure 3A), did not affect either hTERT mRNA or telomerase activity levels. The dissociation between these phenomena has two important implications. First, it demonstrates that upregulation of telomerase activity is neither a prerequisite for proliferation nor its consequence. Second, it indicates that removal of the constraints imposed by growth inhibition is necessary but not sufficient to restore telomerase activity in endothelial cells.

Another major finding of this study was the discovery that Sp1 transcripts are upregulated by FGF-2 but not by VEGF (Figure 4). In other human cell types, Sp1 has been recognized as an essential factor for the activation of hTERT

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The hTERT core promoter region has 5 binding sites for Sp1, which, if inactivated, allow only marginal transactivation of hTERT by c-Myc. In agreement with this model of hTERT regulation, our results can be interpreted as follows. First, during exponential growth in complete medium Sp1, c-Myc and Max are all maximally induced, enabling hTERT transactivation. Second, in contrast, during quiescence, Sp1 and, to a lesser extent, c-Myc are downregulated, turning off hTERT transcription. Third, stimulation of quiescent HUVECs with FGF-2 reinduces Sp1 and c-Myc, and consequently hTERT mRNA expression and telomerase activity are upregulated. Fourth, although VEGF induces c-Myc, it does not induce Sp1 and hence cannot restore telomerase activity. Fifth, the partial upregulation of Sp1 transcripts by FGF-2 may additionally explain why telomerase is not completely restored under these conditions. Lastly, it is noteworthy that Mad1, a repressor of hTERT expression, showed stable mRNA levels under all culture conditions, consistent with its tumor suppressor function and explaining the comparatively low levels of telomerase activity found in normal endothelial cells compared with malignant tumor cells, where Mad1 mRNA is mostly undetectable.

The fact that FGF-2 did not upregulate telomerase activity as effectively as a combination of growth factors providing full mitogenic stimulation suggests that additional mechanisms are involved in the control of this enzyme in endothelial cells. This is also consistent with the finding that depletion of FGF-2 from complete medium caused only a partial reduction in the stimulatory effect (Figure I). Whether these additional mechanisms operate transcriptionally or posttranscriptionally remains to be established. In this context, it is noteworthy that VEGF, which is known to activate the phosphoinositol 3-kinase/Akt/NO synthase pathway, showed no effect on telomerase activity when used in combination with FGF-2, even though NO12 and the protein kinase Akt13 have both been implicated in the regulation of telomerase activity in HUVECs.

In this work, we have shown that cultures grown in the presence of VEGF alone, in contrast to FGF-2, rapidly accumulate cells displaying a senescent phenotype (online data supplement, Figure II). This suggests that the upregulation of telomerase activity by FGF-2 plays a functional role in preventing the early onset of senescence. Furthermore, this finding is consistent with results from other laboratories showing that FGF-2 prolongs the life span of endothelial cells in culture20 and that expression of telomerase enables normal human cells, including HUVEC, to delay or escape senescence.30,31 Interestingly, Watanabe et al23 have reported that VEGF delays the onset of senescence in human dermal microvascular endothelial cells. Our findings (Figure III, online data supplement) demonstrate that the same is not true for HUVECs, in which both the replicative life span and the population doubling level at which senescence characteristics appeared were similar in cultures grown in the absence or presence of VEGF. Although this discrepancy may simply be put down to the inherent biological differences between human dermal microvascular endothelial cells and HUVECs,22 several procedural differences may also account for the conflicting results. These include the use of 20% FCS in the culture medium by Watanabe et al,23 while we used 2% FCS and bovine brain extract as a source of FGF-2, and the fact that they did not supplement cultures with 5 ng/mL VEGF until late passages, while instead we used this concentration of VEGF from the onset.

In the telomerase-deficient mouse, early generations, in which telomeres are still very long, exhibit normal angiogenesis, whereas this phenomenon is impaired in late generations, when telomeres have become critically short.32 This might suggest that telomere length rather than telomerase activity is functionally relevant for normal endothelial replication. However, it should be emphasized that telomere biology in humans is fundamentally different from mice, particularly because human telomeres are much shorter. In contrast, in the present study, the effect of FGF-2 in preventing senescence could not be attributed to a decrease in the rate of telomere shortening but is consistent with more recent evidence suggesting that telomerase plays an active role in promoting cell growth in human cells via a mechanism that preserves telomere function independently of telomere length maintenance.3,4 This concept is based on experiments in HUVECs and other endothelial cells ectopically expressing hTERT. These studies showed that telomerase enabled escape from senescence despite the fact that telomere length continued to decrease to a level far below that of normal senescent cells.31 Therefore, in our study, it was not entirely surprising to find no difference in telomere attrition rates between cultures grown with VEGF or FGF-2, even though there were differences in telomerase activity and the onset of senescence between the two conditions. Nonetheless, we cannot rule out completely the possibility that the differential effects of FGF-2 and VEGF on senescence are attributable to some degree of telomerase-mediated telomere length maintenance, which we cannot detect because of the impossibility to maintain cultures through sufficient populations doublings in the absence of FGF-2.

Endothelial cell senescence is being increasingly implicated in vascular pathology.34–36 Whether the upregulation of telomerase by FGF-2 plays a role in delaying this process in vivo is presently unknown. Regenerating endothelial cells express high levels of FGF-2, and levels of expression decrease when these cells cease to replicate.37 Furthermore, systemic administration of FGF-2 after balloon angioplasty accelerates endothelial regeneration and overcomes the cessation of growth.38 Our findings demonstrate that the modulation of telomerase in endothelial cells is determined by specific growth-related events, in which FGF-2 plays a significant part, but the precise role of these events in vivo remains to be elucidated.

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Methods

Induction of replicative senescence

First passage cryopreserved cells were grown in EGM (Biowhittaker) consisting of modified MCDB131 medium supplemented with 10 ng/ml human recombinant epidermal growth factor, 1 µg/ml hydrocortisone, 50 µg/ml gentamycin, 50 ng/ml amphotericin B, 12 µg/ml bovine brain extract, and 2% (v/v) fetal calf serum at 37°C under 5% CO₂/95% air in a humidified incubator. Cells were serially passaged until they reached senescence, as previously described (1). In order to compensate for the decrease in the rate of cell growth that occurs as the cells advance towards senescence culture times between passages (5-9 days) were adjusted so that the cells reached 90% by the day of harvesting. The number of population doublings was calculated using the formula: PD = (ln[number of cells harvested]-ln[number of cells seeded x attachment efficiency]) / ln2. The percentage of SA-β-gal positive cells was determined by microscopic examination and manual counting as previously described (1).

Reverse transcriptase-polymerase chain reaction analysis of RNA

Synthesis of cDNA templates for polymerase chain reaction (PCR) analysis was carried out for 90 min at 37 °C in a final reaction volume of 20 µl containing 2 µg total cellular RNA sample, 1 µM oligo-dT primer, 10 units RNase inhibitor (Sigma-Aldrich, Dorset, UK), 0.5 mM dNTPs, 4 units Omniscript reverse transcriptase (Qiagen, West Sussex, UK) and buffer supplied by the manufacturers of the enzyme. PCR amplifications were
performed in a 25 µl reaction volume containing 1.5 mM MgCl₂, 1.5 units Taq DNA polymerase (Sigma-Aldrich) and specific oligonucleotide primers as follows: *htert* was amplified for 33 cycles from 5 µl cDNA aliquots of the reverse transcriptase reaction using the primers 5’-TGAACCTTGCGGAAGACAGTG-3’ (sense) and 5’-TGTTCACTGCAAATCCAGA-3’ (anti-sense). *Sp1* was amplified for 25 cycles from 2-µl cDNA using the primers 5’-TGCAGCAAGATGTGCCACC-3’ (sense) and 5’-CACAAATCATGCCCCCACCAG-3’ (antisense). *c-Myc* was amplified for 25 cycles from 2 µl cDNA using the primers 5’-CAGCTGCTTAGACGCTGGATTT-3’ (sense) and 5’-ACCGAGTGACTGTCCAGGTCAT-3’ (antisense). *Max* was amplified for 25 cycles from 2 µl cDNA using the primers 5’-TAGTGTCCCTGCCCACCCTAC-3’ (sense) and 5’-AACAGCTGGCTGAGAGAAGC-3’ (antisense). *Mad1* was amplified for 30 cycles from 2 µl cDNA using the primers 5’-AGGAGTGTCAGCGACTCTGA-3’ (sense) and 5’-CCAACAGGGAGAACCCTCCTCA-3’ (antisense). *Glyceraldehyde-3- phosphate dehydrogenase* (*GAPDH*) was amplified for 22 cycles from 2 µl cDNA using the primers 5’-CCTGTTCGACAGTCAGCC-3’ (sense) and 5’-CGACCAAATCCGTTGACTCC-3’ (anti-sense). In every case each cycle consisted of 95°C for 15 s, followed by 60°C for 20 s and then 72°C for 20 s. After amplification 12 µl aliquots of the PCR reactions were separated on 2% agarose gels.

**References**

Legends to online figures

**Figure I: FGF-2 withdrawal down-regulates telomerase activity in cultured HUVEC.** Non-confluent, quiescent HUVEC were incubated for two days with fresh basal medium (BM), fresh EGM-2, fresh EGM-2 without FGF-2, or fresh EGM-2 without FGF-2 with either added FGF-2-neutralizing antibodies (AB) or isotype control antibodies, as indicated. Values represent the mean (± SD) telomerase activity of 3 experiments. *P<0.05, **P<0.01 by ANOVA with Bonferroni’s multiple test correction.

**Figure II: Comparison of SA-β-galactosidase staining in HUVEC cultured with either VEGF or FGF-2.** Second passage cells were grown in basal medium supplemented with either 25 ng/ml FGF-2 or 25 ng/ml VEGF and then sub-cultured two more times when they reached ~75% confluence or after two weeks in culture. Photomicrographs show SA-β-gal staining after 10 days into the fifth passage. Note the increase in cell size in VEGF-maintained cultures. Magnification = 100X.

**Figure III: Effect of VEGF supplementation on replicative life span of HUVEC.** First passage HUVEC were grown to ~90% confluence in EGM in the absence (full circles) or presence (empty circles) of 5 ng/ml VEGF. Cultures were serially passaged until the onset of senescence. Graphs show (A) cumulative population doublings (CPD) and (B) percentage of SA-β-gal positive cells as a function of time in culture.
Figure I

Telomerase activity, AU

BM
EGM-2
EGM-2 w/o FGF-2
+ FGF-2 neutr. AB
+ isotype control AB

*  
**
Figure III

Panel A: CPD over days with and without 5 ng/ml VEGF.

Panel B: % SA-β-gal+ cells over days with and without 5 ng/ml VEGF.