Telomerase Mediates Vascular Endothelial Growth Factor-dependent Responsiveness in a Rat Model of Hind Limb Ischemia*

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Telomere dysfunction contributes to reduced cell viability, altered differentiation, and impaired regenerative/proliferative responses. Recent advances indicate that telomerase activity confers a pro-angiogenic phenotype to endothelial cells and their precursors. We have investigated whether telomerase contributes to tissue regeneration following hind limb ischemia and vascular endothelial growth factor 165 (VEGF$_{165}$) treatment. VEGF delivery induced angiogenesis and increased expression of the telomerase reverse transcriptase (TERT) and telomerase activity in skeletal muscles and satellite and endothelial cells. Adenovirus-mediated transfer of wild type TERT but not of a dominant negative mutant, TERTdn, significantly induced capillary but not arteriole formation. However, when co-delivered with VEGF, TERTdn abrogated VEGF-dependent angiogenesis, arteriogenesis, and blood flow increase. This effect was paralleled by *in vitro* evidence that telomerase inhibition by 3′-azido-3′-deoxythymidine in VEGF-treated endothelial cells strongly reduced capillary density and promoted apoptosis in the absence of serum. Similar results were obtained with adenovirus-mediated expression of TERTdn and AKTdn, both reducing endogenous TERT activity and angiogenesis on Matrigel. Mechanistically, neo-angiogenesis in our system involved: (i) VEGF-dependent activation of telomerase through the nitric oxide pathway and (ii) telomerase-dependent activation of endothelial cell differentiation and protection from apoptosis. Furthermore, detection of TERT in activated satellite cells identified them as VEGF targets during muscle regeneration. Because TERT behaves as an angiogenic factor and a downstream effector of VEGF signaling, telomerase activity appears required for VEGF-dependent remodeling of ischemic tissue at the capillaries and arterioles level.

Vascular endothelial growth factor (VEGF)[1] is a potent and cell-specific angiogenic factor, which enables the formation of new vascular structures in normal (1) and ischemic tissues (2). Recently, the VEGF range of cellular targets has been potentially extended beyond endothelial cells because its receptors are expressed in other tissues (3, 4). Specifically, the presence of VEGF receptor 2 (VEGFR-2) in regenerating muscle fibers and skeletal muscle satellite cells (3, 5) suggests a specific action of VEGF on these cells during post-ischemic tissue regeneration and gene therapy interventions (4). Beyond evidence that VEGF multiple signaling pathways regulate proliferation, migration, and differentiation of endothelial cells (6), little is known about the mechanism(s) by which VEGF promotes angiogenesis *in vivo* in normal or ischemic tissues. A growing body of literature assigns to telomerase a potentially relevant role in angiogenesis and cardiovascular disorders (7, 8). To date, however, no evidence has linked VEGF and the regulation of the endogenous catalytic subunit (TERT) of telomerase, which is limiting for enzymatic activity.

Here, we have investigated the role of telomerase in tissue remodeling after VEGF gene transfer in a rat model of hind limb ischemia. We found that telomerase is an important downstream effector of VEGF-mediated vascularization *in vivo* involved in the regulation of capillarogenesis. In fact, adenovirus-mediated transfer of the wild type hTERT gene in ischemic rats induced development of new capillaries and reduced apoptosis, indicating a direct contribution of TERT to angiogenesis *in vivo*. Telomerase activity is required for this process, because delivery of a dominant negative mutant of TERT (TERTdn) failed to promote formation of new capillaries. Most strikingly, when co-delivered with VEGF, the TERTdn completely abrogated VEGF-dependent angiogenesis and arteriogenesis, emphasizing the role of the enzyme in the vascular system. Similarly, the inhibition of endogenous telomerase by 3′-azido-3′-deoxythymidine (AZT) in VEGF-treated cells strongly reduced

[1] The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; hTERT, human TERT; dn, dominant negative; AZT, 3′-azido-3′-deoxythymidine; NO, nitric oxide; NOS, nitric-oxide synthase; eNOS, endothelial NOS; bFGF, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cell(s); Ad, adenovirus; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; PI3K, phosphatidylinositol 3-kinase; ANOVA, analysis of variance; RT, reverse transcription; 7N, 7-nitroindazole; ERK, extracellular signal-regulated kinase; aFGF, acidic fibroblast growth factor; TRAP, telomeres repeat amplification protocol; TUNEL, TdT-mediated dUTP nick end labeling; TERT, telomerase reverse transcriptase.

capillary formation and failed to prevent apoptosis in the absence of growth factors. In addition, the expression of TERTdn or AKTdn reduced the catalytic activity of endogenous telomerase and severely compromised endothelial cell differentiation on Matrigel, supporting a further role of TERT in the regulation of endothelial cell differentiation. Lastly, we obtained evidence as to the mechanism involved in VEGF-dependent angiogenesis by demonstrating that: (i) interference with the VEGF/VEGFR-2 pathway by inhibition of nitric oxide (NO) synthesis prevented the increase of *TERT* mRNA and telomerase activity in differentiated C2C12 myotubes and in proliferating human umbilical vein endothelial cells (HUVEC) and (ii), conversely, the expression of a constitutively active eNOS mutant activated the *TERT* promoter.

MATERIALS AND METHODS

Reagents and Antibodies—Human recombinant VEGF$_{165}$, aFGF, and fibroblast growth factor (bFGF) (R&D System), 7-nitroindazole (Santa Cruz Biotechnology), PD098059 (Biomol), SB202190 (Alexis Biochemical), and AZT (Sigma) were obtained. Anti-TERT K370 (Calbiochem), LY-294002 (Biomol), anti-hTERT rabbit polyclonal antibody (a gift from Lea Harrington, University of Toronto), anti-Desmin (Dako), goat anti-rabbit-biotinylated secondary antibodies unconjugated or conjugated with fluorescein or Texas Red (Vector Laboratories) were also used.

Animal Studies—Wistar male rats (Harlan) (3–4-months-old, 300–350 g) were used. Unilateral hind limb ischemia was induced as described previously (1) following a protocol approved by the Institutional Animal Care and Use Committee.

Adenovirus Vectors—Replication-deficient recombinant adenovectors were prepared, stored, and administered as described previously (1, 9). In all of the vectors, E1 was replaced by the cytomegalovirus-immediate early promoter region gene promoter/enhancer driving the cDNA for human VEGF$_{165}$ or LacZ gene (9), the hTERT or hTERTdn cDNA (10), or no gene (AdNull) (1).

Gene Therapy Protocol—Sham-operated and ischemic rats, immediately after femoral artery removal, were randomly assigned to receive intramuscular injection of AdNull, AdLacZ, or AdVEGF$_{165}$ at four sites on the thigh along the projection of the femoral artery. AdhTERT and AdhTERTdn or saline were injected at 10 sites. For all of the experiments, the virus dose was 5×10^7 pfu/animal in 0.5 ml except as indicated when a combination of 5×10^7 pfu/virus for a total of 1×10^8 pfu/animal was used.

Histological and Morphometric Analysis—Animals (4–8/time point) were anesthetized and perfused via the left ventricle with 10% formaldehyde at 100 mm Hg for 10 min. Adductor muscles were removed, fixed in formaldehyde for 48 h, and embedded in paraffin. For each sample, 3-μm-tick sections were stained with hematoxylin-eosin and 90–130 random fields were examined to evaluate capillary density as described previously (1, 11, 12).

Telomerase Assay—Extracts were prepared by detergent lysis followed for tissues by mechanical homogenization and assaying without freezing. Telomerase activity was detected by TRAP (13).

Arteriole Length Density—Arteriole formation was determined as described previously (1, 12). After paraffin embedding, 3-μm sections from each sample were cut with the muscle fibers oriented in the transverse direction. Thereafter, sections were de-paraffinized, rinsed in PBS, incubated at 37 °C for 60 min with mouse monoclonal anti-α-smooth muscle actin (clone 1A4, Sigma) diluted in 1:30 PBS, and incubated at 37 °C for 60 min with anti-mouse IgG TRITC-labeled antibody diluted 1:60 in PBS. Sections were then rinsed in PBS and embedded in Vectashield (Vector Laboratories) mounting medium. For morphometric analysis, the total area of each section was examined at $\times 400$ magnification. In each field, the measurement of arteries and arteriole profile included major and minor luminal diameter length. Specifically, for n profiles counted in an area (A), the length density (Ld) is equal to the sum of the ratio (R) of the major or long axis to the minor or wide axis of each profile according to the formula: Ld = $1/A \times \Sigma^R$ = $(R1 + R2 + R3 + \ldots \ldots Rn)/A$.

Ultrasonic Transit Time Flowmetry—Blood flow was measured by ultrasonic transit time flowmetry using a Transonic flowmeter (T 106, Transonic Systems Inc.) coupled to a 100-Hz 0.5-V probe calibrated according to the manufacturer's instructions. Flowmetry was performed on the residual part of the femoral artery underlying the inguinal ligament in the ischemic limb and on an equivalent portion of the controlateral normoperfused limb. The probe was directly applied on the artery, which was not deflected from its natural course. Space between the vessel and the brackets of the probe was filled with an ultrasonic couplant (HR Lubricating Jelly). Blood flow (ml/min) was measured in both controlateral and ischemic limbs and was expressed as ischemic/controlateral ratio. Data are expressed as the mean $\pm$ S.D. Four animals were used for each experimental condition.

Cell Culture and Transfection—The murine myoblast C2C12 cell line was grown, transfected, and differentiated as described previously (14). Recombinant VEGF$_{165}$, aFGF, and bFGF were added 24 h after induction of differentiation. The p3996-hTERT promoter vector has been described previously (15). The eNOS vector encoding eNOS S1177D was provided by S. Dimmeler (16). HUVEC were cultured according to the supplier's protocols (Cambrex). Bovine vein endothelial cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone). 7-Nitroindazole, PD098059, LY-294002, or SB202190 was added 1 h prior to treatment with angiogenic factors.

Proliferation Rate and Cell Cycle Analysis—Cell proliferation was determined by plating 0.5×10^4 cells in octuplicate in 96-well plates. Cell viability was assessed by colorimetric assay using Cell Titer-Blue (Promega). Cell cycle profiles were evaluated by fixing 5×10^5 cells in 4% paraformaldehyde and 0.1% Triton X-100 for 10 min on ice and staining DNA for 30 min at room temperature with 50 μg/ml propidium iodide in PBS containing 1 mg/ml RNase A. Percentages of sub-G$_1$ cells were measured by flow cytometric analysis of propidium iodide-stained nuclei using Multicycle Software (Phoenix Flow System), Epics XL (Coulter).

Immunofluorescence—C2C12 and HUVEC were immunostained as described previously (15). For satellite cells, tissue sections, which were de-paraffinized, re-hydrated, PBS-washed, and microwaved for 20 min, were incubated for 1 h at room temperature with 2% bovine serum albumin and overnight at 4 °C with primary antibody. After washing twice with PBS, slides were incubated with fluorescein- or Texas Red-conjugated secondary antibody.

Differentiation Assay—Experiments were performed in 24-multiwell plates coated with 400 μl/well Matrigel as described previously (17). 5×10^4 cells/well were seeded with or without VEGF (50 ng/ml) in the presence or absence of AZT (100 μM). The number of tubular structures from triplicate wells (10 fields/well) was quantified at $\times 20$ magnification after 3 h of differentiation. Each experiment was performed in triplicate.

TUNEL Assay—All of the procedures were performed as previously described (5). Cells or muscle sections were incubated with 5 units of terminal deoxynucleotidyltransferase, 2.5% mM CoCl$_2$, 0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.25% bovine serum albumin, and 0.5 nM biotinylated 2'-dUTP (biotin-16-dUTP). Samples were incubated with avidine-biotin complex-rhodamine or avidine-biotin complex-horseradish peroxidase complex and revealed with 3'-diamino-benzidine. HUVEC were plated in the absence of growth factors, and VEGF in combination or not with AZT was added to the medium. After 36–48 h, cells were harvested and cytocentrifuged onto glass slides. A total of 20 randomly chosen fields were counted for each slide, and total counts were averaged to obtain the apoptotic index.

Differentiation Assay—Experiments were performed in 24-multiwell plates coated with 400 μl/well Matrigel (BD Biosciences) as described previously (17). 5×10^4 cells/well were seeded with or without VEGF (50 ng/ml) in the presence of AZT (10 and 100 μM) or AZT (10 μM and 100 μM) heat-inactivated for 10 min at 95 °C. Infection of HUVEC with recombinant adenovirus was performed in PBS$^{+/+}$ for 45 min at room temperature, and the cells were harvested and seeded on Matrigel. The number of tubular structures from triplicate wells (10 fields/well) was quantified at $\times 20$ magnification after 3–24 h of differentiation. Each experiment was performed in triplicate.

Statistical Analysis—Continuous variables were analyzed by the Student's t test and one-way ANOVA. Post hoc tests according to the Student-Newman-Keul's method were used when the ANOVA p value indicated a statistically significant difference among groups. Data are expressed as the mean $\pm$ S.E. A value of $p < 0.05$ was deemed statistically significant.

RESULTS

VEGF$_{165}$ Induces Angiogenesis and Up-regulates TERT in Ischemic Rats—Unilateral hind limb ischemia was induced by removal of the femoral artery in male Wistar rats. Consequences of hind limb ischemia in this model have been well characterized (1). Gene therapy treatment was administered at the time of surgery by intramuscular injection of an adenovector encoding the human isoform of VEGF$_{165}$ (AdVEGF$_{165}$) into
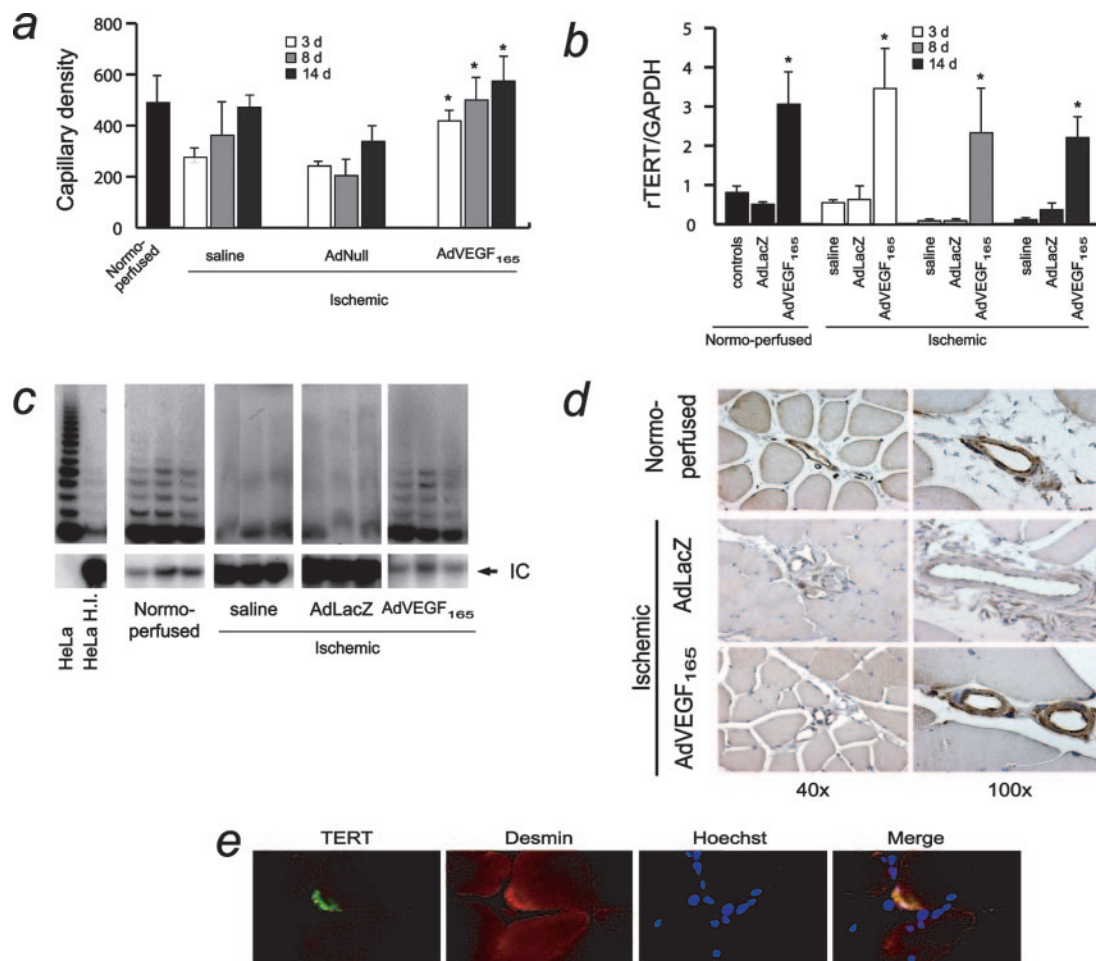
FIG. 1. **VEGF induces angiogenesis and up-regulates TERT expression and telomerase activity *in vivo.*** Normoperfused or ischemic rat adductor muscles upon intramuscular injection of saline (*saline*), a control adenoviral vector (*AdNull*), or an adenoviral vector carrying the human isoform of VEGF$_{165}$ (*AdVEGF$_{165}$*) were collected at 3, 8, and 14 days after injection. *a*, capillary density was evaluated on hematoxylin-eosin-stained sections. Values were expressed as counts per mm^2. *b*, expression of *rTERT* mRNA was analyzed by RT-PCR. Densitometric analysis of *rTERT* levels was performed after normalization to values obtained under the same conditions with the control housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Statistical significance (AdVEGF$_{165}$ *versus* AdNull/AdLacZ) $p < 0.05$ is indicated (*asterisk*). *c*, telomerase activity was measured by TRAP assays. The results shown are from 3 randomly chosen rats of 12 after 3 days of treatment. HeLa and HeLa heat-inactivated (*H.I.*) extracts were used as positive and negative controls, respectively. *IC*, the internal standard. *d*, expression of TERT protein in skeletal muscles and vascular structures. Normoperfused and ischemic tissues were immunostained for TERT and counterstained with hematoxylin. Representative panels from ischemic rats are shown at 3 days after gene therapy. *e*, immunofluorescence of skeletal muscle satellite cells. Sections from ischemic tissues after VEGF$_{165}$ treatment were stained with anti-TERT (*green*) and anti-desmin (*red*) antibodies for analysis of satellite cells. Fluorescence microscopy showed that desmin-positive activated satellite cells (localized at the edges of skeletal muscles cells where regeneration starts) co-express TERT (*Merge*). Nuclei were stained with Hoechst 33258 (*blue*).

the adductors and quadriceps of normoperfused (sham-operated) or ischemic rats. Control animals received saline, AdNull, or AdLacZ. As shown in Fig. 1*a*, VEGF$_{165}$ gene delivery enhanced capillary density. In treated rats, values were significantly higher than in controls (AdNull) at each time point (3 days, $p < 0.0001$; 8 days, $p < 0.045$; and 14 days, $p < 0.032$).

A large body of literature indicates that VEGF regulates the function of mature endothelial cells and mobilizes vascular precursor cells (18). VEGF also protects vascular cells from apoptosis (19) and stimulates angiogenesis in normal (1) and ischemic skeletal muscles (20). However, the mechanisms underlying this vascularization remain elusive (21). Recent evidence shows that a sustained telomerase activity improves cell survival (22) and enhances the angiogenic properties of endothelial cells and their precursors (23–25). No cooperative angiogenic effect between VEGF and endogenous TERT has been reported to date. We sought to evaluate whether VEGF$_{165}$ modulates TERT expression and telomerase activity during a time course of 3, 8, and 14 days in rat skeletal muscles. In the untreated animals, *rTERT* mRNA expression was low (26, 27)

and decreased further after ischemia (Fig. 1*b*) but was markedly enhanced upon VEGF$_{165}$ gene delivery at all of the time points ($p < 0.01$). *rTERT* mRNA did not increase upon injection of control virus (AdLacZ). Telomerase activity paralleled changes in mRNA level (Fig. 1*c*). It was low but detectable in the skeletal muscles of normoperfused animals and virtually absent following ischemia but substantially rescued by 3 days of treatment with VEGF$_{165}$.

Expression of the TERT protein was detected (Fig. 1*d*) in muscle fibers and vascular structures of normoperfused animals but was down-regulated upon ischemia and again substantially rescued by VEGF$_{165}$ treatment. VEGFRs have been recently detected in non-vascular tissues including regenerating skeletal muscle fibers and satellite cells (5, 16). To investigate a potential correlation between VEGF activation of satellite muscle cells and of TERT expression, serial muscle sections were immunostained with antibodies for TERT or desmin, a specific marker of activated satellite cells (see Ref. 5 and references therein). Satellite cells of ischemic rats at day 3 after VEGF treatment did co-express TERT and desmin (Fig.

FIG. 2. **Adenovirus-mediated gene transfer of hTERT promotes angiogenesis and protects from apoptosis in ischemic rats.** Ischemic rat adductor muscles were injected intramuscularly with saline (*saline*), AdNull, AdVEGF$_{165}$, AdhTERT, or AdhTERTdn or a combination of AdVEGF and AdhTERTdn and collected at the indicated times. *a*, *hTERT* levels in infected animals were measured by RT-PCR using primers specific to *hTERT*. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as control. The results shown are from two randomly chosen rats after 3, 8, and 14 days of treatment. *b*, capillary density evaluated on hematoxylin-eosin-stained sections is expressed as counts per mm^2. The effect of TERTdn was evaluated at the time (8 days) when the response to VEGF$_{165}$ treatment was maximal compared with controls (see Fig. 1*a*). *c*, arteriole formation was evaluated on ischemic rats 14 days after gene therapy and expressed as length density per mm^3. *d*, blood flow of normoperfused and ischemic tissues was evaluated by ultrasonic transit time flowmetry 14 days after gene therapy treatment. *e*, TUNEL assays were performed on paraffin sections of ischemic rats 3 days after treatment. Values are expressed as TUNEL-positive nuclei per mm^2. Statistical significance is indicated as follows: *asterisk*, AdVEGF$_{165}$ or AdhTERT *versus* AdNull $p < 0.02$; *§*, AdVEGF$_{165}$ + AdhTERTdn *versus* AdVEGF$_{165}$ $p < 0.0006$; #, 1×10^8 pfu.
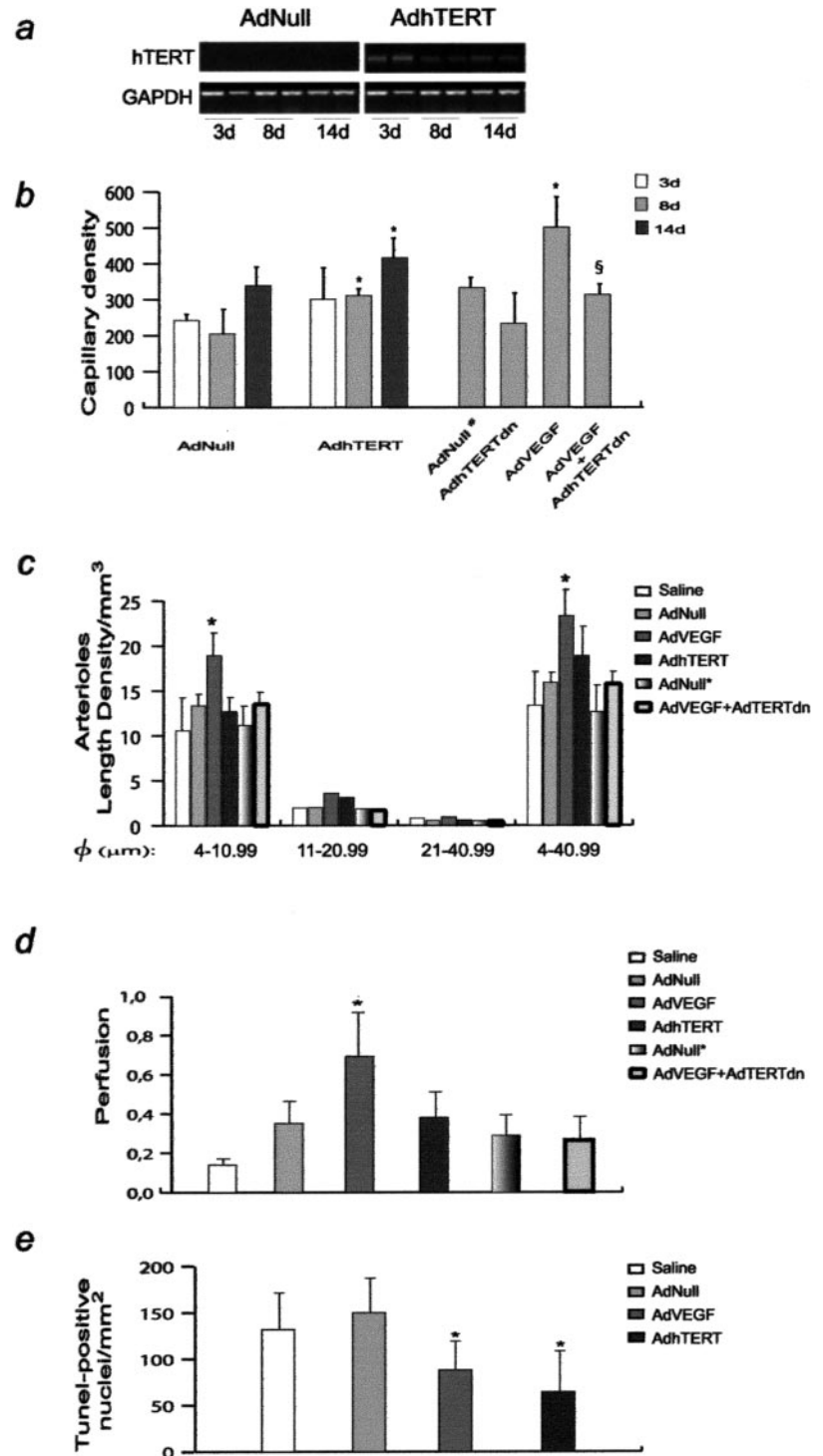
1*e*), establishing a correlation between telomerase activation and muscle regeneration. Satellite cells in normoperfused or ischemic animals receiving saline or Ad.Null were negative for both markers (data not shown). These results indicate that VEGF$_{165}$ gene therapy of hind limb ischemia induces TERT expression and function and that this effect is concomitant with the onset of an angiogenic process.

Telomerase Induces Angiogenesis in Ischemic Tissues and Protects Them from Apoptosis—We next queried whether the activation of telomerase is essential for VEGF$_{165}$-dependent angiogenesis in acute hind limb ischemia or is an epiphenomenon devoid of biological significance. To address this point, we examined *in vivo* the effect of adenovirus-mediated transfer of

the hTERT gene on the formation of blood vessels and on blood flow in rat ischemic muscles at 3, 8, and 14 days after injection (Fig. 2). The expression of the transduced gene was monitored by RT-PCR using primers specific for *hTERT* (Fig. 2*a*). No induction of the endogenous *TERT* gene was detected upon infection with AdNull using primers specific for the rat molecule (data not shown). Notably, compared with AdNull, capillary density was enhanced by AdhTERT (Fig. 2*b*), particularly by 14 days. However, the angiogenic effect of hTERT was more limited than that of VEGF. No significant increase was detected in the formation of arterioles (Fig. 2*c*) and on blood flow (Fig. 2*d*) in ischemic animals infected with AdhTERT, whereas both parameters were enhanced by VEGF. In addition, when
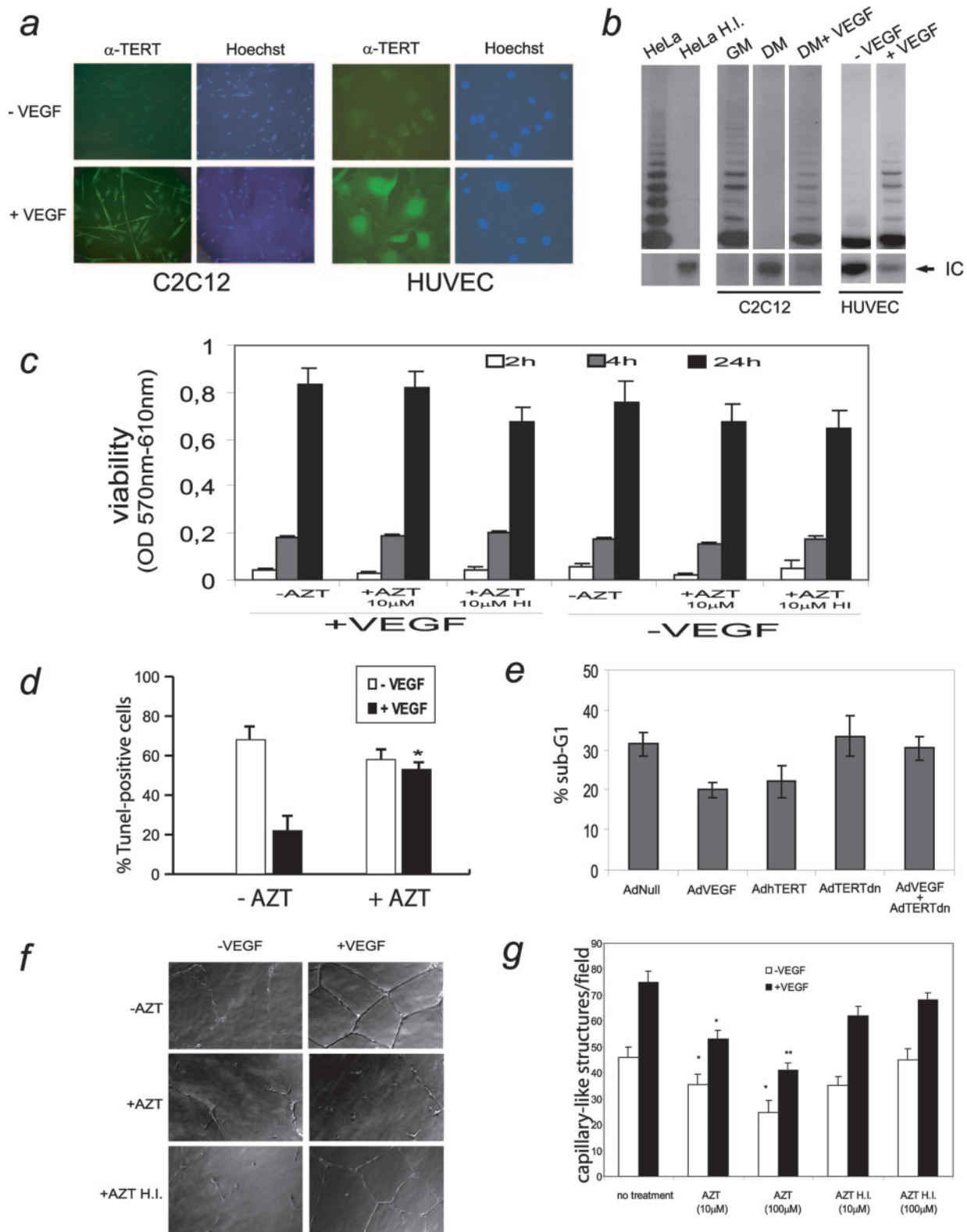
FIG. 3. **The induction of telomerase activity is necessary for VEGF-dependent angiogenesis in *vitro*.** *a*, differentiated C2C12 or proliferating HUVEC were grown with or without recombinant VEGF$_{165}$ (50 ng/ml) and stained with an anti-TERT antibody (*α-TERT*) or with Hoechst 33258. Magnification is ×10 for C2C12 and ×40 for HUVEC. *b*, telomerase activity was assayed in extracts from proliferating (*GM*) and differentiated (*DM*) C2C12 cells and in HUVEC in the absence (−) or presence (+) of recombinant VEGF$_{165}$. Positive and negative controls and internal standard are as shown in Fig. 1. *c*, viability of HUVEC cultured in the presence or absence of VEGF and with or without AZT (10 and 100 μM) or heat-inactivated AZT (10 μM and 100 μM) at the indicated time. *d*, HUVEC cultured on Matrigel in the presence or absence of VEGF and with or without AZT. *e*, sub-G$_1$ percentage of HUVEC cultured in the absence of growth factors for 48 h after adenoviral infection obtained by cytofluorimetric analysis after propidium iodide staining. *f*, HUVEC cultured on Matrigel in the presence or absence of VEGF and with or without AZT (10 μM) or heat-inactivated AZT (+*AZT H.I.*, 10 μM). *g* and *h*, quantification of capillary-like structures in each condition is represented as the mean ± S.E. of 10 randomly chosen fields as described under "Material And Methods." *Crtl*, control. *i*, telomerase activity in VEGF-treated HUVEC was evaluated in each condition by TRAP. Positive and negative controls and internal standard are as described in Fig. 1.
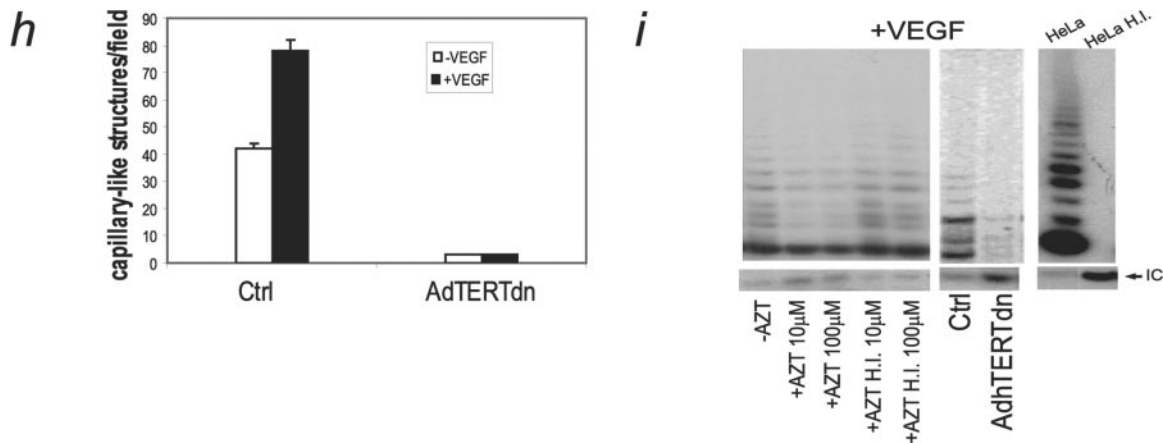
F<small>IG</small>. 3—*continued*

VEGF was co-delivered with a dominant-negative mutant hTERT (AdhTERTdn), which on its own has no effect on capillary density, VEGF-dependent effects on angiogenesis (Fig. 2*a*), arteriogenesis (Fig. 2*b*), and blood flow (Fig. 2*c*) were abrogated. On the other hand, wild type hTERT was as efficient as or even more so than VEGF in protecting cells from apoptosis (Fig. 2*e*). All of these observations demonstrate a direct contribution of TERT to the onset of new vessels promoted by VEGF treatment.

Telomerase Activity Is Required for the Onset of VEGF-dependent Angiogenesis in Vitro—To further examine the role of TERT in angiogenesis, we measured TERT expression and telomerase activity in differentiated murine myotubes (C2C12) and HUVEC grown in the absence or presence of 50 ng/ml human recombinant $VEGF_{165}$ (Fig. 3). As expected (24, 28), endogenous TERT was expressed at negligible levels in differentiated C2C12 or proliferating HUVEC, as assessed by indirect immunofluorescence with a TERT antibody (Fig. 3*a*). $VEGF_{165}$ strongly induced TERT mRNA expression in both cell types with peak expression at 20–24 h (data not shown). This effect was paralleled by a significant increase in telomerase activity (Fig. 3*b*). To analyze functionally the role of hTERT during VEGF-dependent endothelial cell survival and *in vitro* differentiation, a series of experiments were performed in which hTERT activity was inhibited by AZT (29) or TERTdn expression (Fig. 3, *c–i*). Fig. 3*c* shows that, in the presence of serum, AZT does not significantly compromise endothelial cells viability upon treatment for 2–24 h. However, in the absence of other growth factors, $VEGF_{165}$ pro-survival effects were markedly reduced in cells exposed to AZT (Fig. 3*d*) or infected with TERTdn (Fig. 3*e*).

Remarkably, AZT-treated or TERTdn-infected HUVEC cultured in the presence or absence of VEGF failed to form VEGF-dependent capillary-like structures on Matrigel (Fig. 3, *f–h*). Under the same conditions, both AZT and TERTdn severely compromised telomerase activity (Fig. 3*i*), thus indicating that a functional enzyme may be required for $VEGF_{165}$ differentiation and pro-survival effects.

$VEGF_{165}$ Treatment Induces TERT Expression and Telomerase Activity via PI3K/AKT and Nitric Oxide—It has been reported that VEGF regulates NO production via PI3K/AKT signaling (30), which may account for some of the VEGF-dependent vascular protective effects (19). Fig. 4*a* shows that PI3K signaling is important for *hTERT* expression, which is significantly reduced by the PI3K inhibitor LY294002. Consistently, in VEGF-stimulated endothelial cells, the inhibition of PI3K dramatically reduced VEGF-dependent telomerase activity (Fig. 4*b*) and transcription from the human *TERT* promoter (Fig. 4*c*). The regulation of telomerase expression and function

via PI3K signaling prompted us to evaluate whether this pathway could be relevant for endothelial cell differentiation. Fig. 4*d* shows that endothelial cells in which a dominant negative AKT molecule has been overexpressed by means of adenovirus vector exhibit significant reduction in TERT activity, which is paralleled by a reduction in capillary-like structure formation (Fig. 4*e*). Therefore, we investigated the role of nitric-oxide synthases (NOS) on *TERT* mRNA expression (Fig. 5*a*), telomerase activity (Fig. 5*b*), and *TERT* promoter transcriptional activity (Fig. 5*c*). Differentiated C2C12 and proliferating HU-VEC were treated with the NOS inhibitor, 7-nitroindazole (7N), in the presence or absence of different angiogenic factors. Recombinant VEGF, aFGF, or bFGF reproducibly induced *TERT* mRNA (1.5–3-fold the unstimulated level) and telomerase activity (Fig. 5, *a* and *b*), revealing a commonality of action of these factors and underscoring the correlation between promotion of angiogenesis and telomerase regulation. Of note, preincubation of cells with 7N abolished only $VEGF_{165}$-dependent effects, indicating that acidic and basic FGF operate via mechanisms that do not involve NO. Preincubation with an inhibitor of p38 mitogen-activated protein kinase pathway (SB203580) had no effect on *hTERT* mRNA levels (Fig. 5*a*). Furthermore, $VEGF_{165}$ activated the hTERT promoter in transient transfection assays (Fig. 5*c*), an effect again abrogated by 7N but not by PD098059 (PD), an antagonist of ERK mitogen-activated protein kinase signaling pathway. The role of NO in the VEGF-dependent transcriptional regulation of *TERT* is further supported by the observation that transfection of a phosphomimetic constitutively activated eNOS mutant (16) in the absence of VEGF up-regulates the *hTERT* promoter to levels similar to those obtained with VEGF alone (Fig. 5*c*).

All of the above findings are consistent with our *in vivo* data indicating that VEGF is capable of inducing TERT expression and telomerase activity in differentiated skeletal muscles and vessels. Furthermore, they reveal that PI3K signaling pathway, which regulates NO synthesis and NO itself, is involved in this process.

DISCUSSION

VEGF induces angiogenesis in a variety of tissues by a mechanism involving activation of a signaling cascade in cells expressing the two VEGF receptors. Recently, the expression of VEGFR-1 and VEGFR-2 has been detected also in non-vascular cells such as regenerating muscle fibers and satellite cells (3, 5), suggesting that some non-endothelial cells may be targeted by VEGF during physiologic angiogenesis and angiogenesis accompanying tumor growth or $VEGF_{165}$ treatment of ischemia. Skeletal muscle tissue frequently undergoes degeneration after abrupt interruption of blood flow (31) but can regenerate
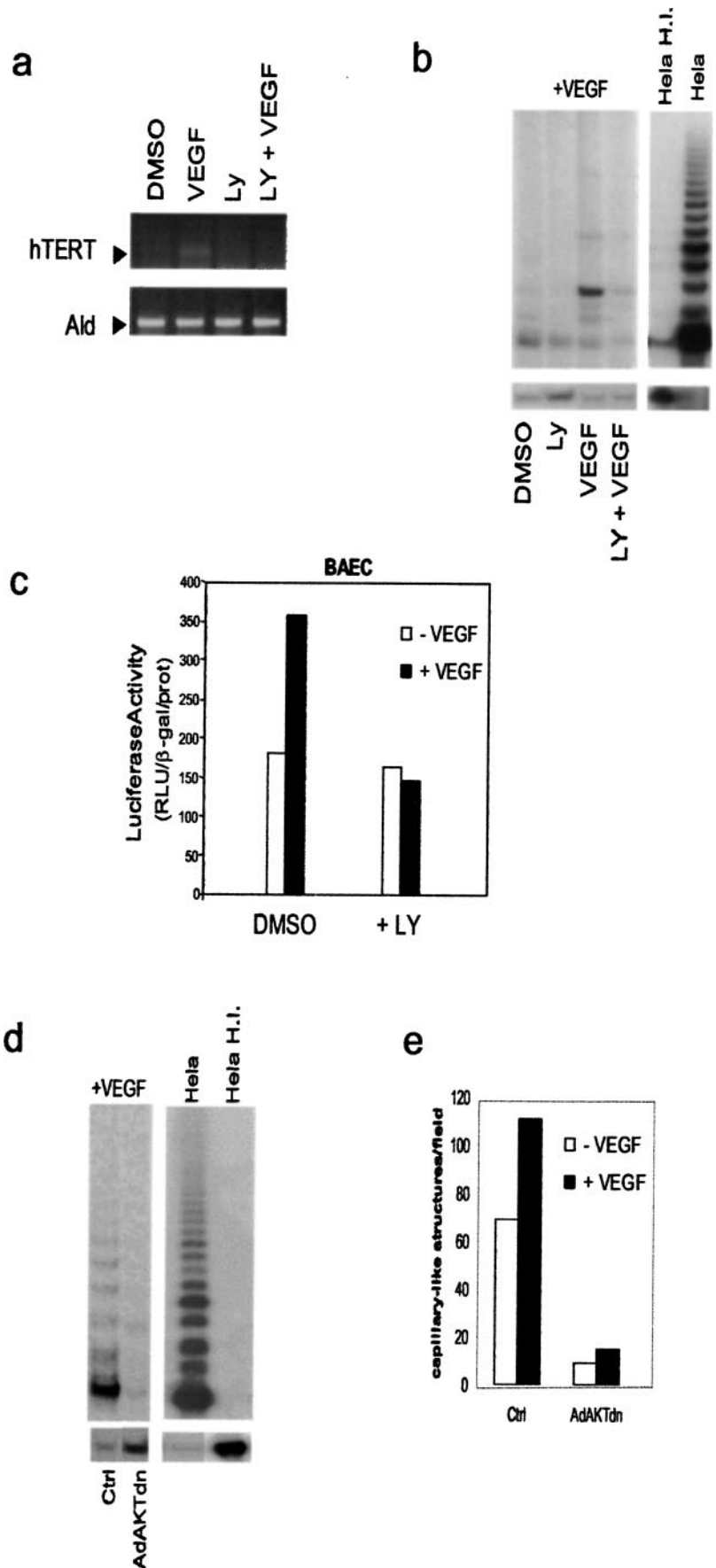
a

hTERT ▶

Ald ▶

DMSO | VEGF | Ly | LY + VEGF

b

+VEGF

Hela H.I. | Hela

DMSO | Ly | VEGF | LY + VEGF

c

BAEC

□ – VEGF
■ + VEGF

d

+VEGF | Hela | Hela H.I.

Ctrl | AdAKTdn

e

□ – VEGF
■ + VEGF

Ctrl | AdAKTdn

FIG. 4. **PI3K/AKT pathway mediates VEGF-dependent telomerase induction in endothelial cells.** *a*, RT-PCR analysis of *hTERT* expression in HUVEC cultured in the presence or absence of recombinant $VEGF_{165}$ and the PI3K inhibitor LY-294002 (*LY*). *DMSO*, Me_2SO. *b*, telomerase activity in the same experimental condition of *panel a*. *H.I.*, heat-inactivated. *c*, transient transfection of analysis of the *hTERT* promoter reporter construct p3996 containing a 3996-bp fragment of the *hTERT* promoter linked to the luciferase reporter gene. The experiment was performed in bovine endothelial cells (*BAEC*) in the presence or absence of recombinant $VEGF_{165}$. Results are representative of three independent experiments each performed in duplicate. *d*, telomerase activity detected by TRAP assay in cells infected with AdAKTdn. *e*, quantification of capillary-like structures longer than three cells. The graph is represented as the mean ± S.D. of 10 randomly chosen fields as described under "Material and Methods."

rapidly upon activation of satellite cells (32). Interestingly, the production of VEGF increases during muscle regeneration following ischemia (33), although the molecular mechanisms underlying this phenomenon are largely undefined. Here, we have shown that treatment with adenovirus-expressing $VEGF_{165}$ induces revascularization of rat ischemic adductor muscles.
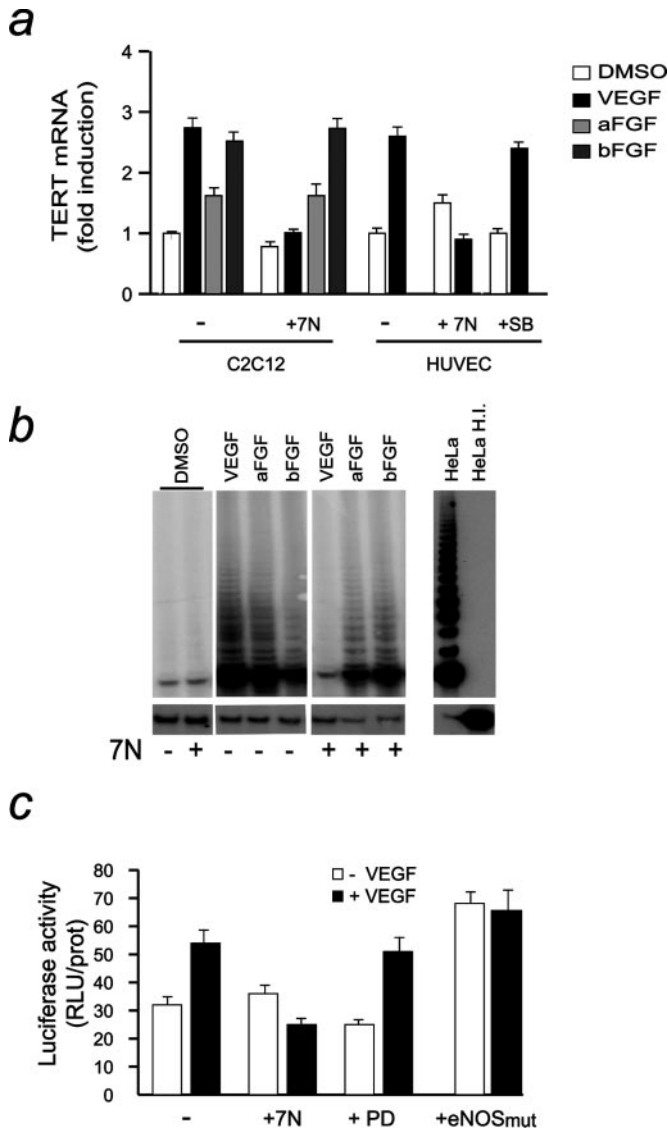
FIG. 5. **VEGF$_{165}$ treatment induces mouse *TERT* RNA expression, telomerase activity, and promoter transcription via nitric-oxide synthases**. Differentiated C2C12 and HUVEC, grown in the presence or absence of angiogenic factors (VEGF, aFGF, and bFGF) or solvent alone (Me$_2$SO, *DMSO*), were preincubated with (+) or without (−) the nitric-oxide synthases inhibitor, 7N, or a p38 inhibitor SB 203580 (*SB*). *a*, *TERT* mRNA levels were measured by densitometry, and values were normalized to those of the control housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The average ratios (TERT/glyceraldehyde-3-phosphate dehydrogenase) from three independent experiments are expressed as fold induction (±angiogenic factor). *b*, telomerase activity in treated C2C12 cells was assayed by TRAP in the presence of the internal standard (*IC*). Positive and negative controls are as in Fig. 1. *H.I.*, heat-inactivated. *c*, transient co-transfections of vector p3996 containing a 3996-bp fragment of the *hTERT* promoter linked to the luciferase reporter and eNOS encoding a mutant NO synthase were performed in proliferating C2C12 cells. After transfection, differentiation was induced and recombinant VEGF$_{165}$ was added to the medium in the presence or absence of the NOS inhibitor (7N) or an antagonist of mitogen-activated protein kinase PD98059 (*PD*). Results represent the average (± S.E.) of three independent experiments, each performed in duplicate.

This process is paralleled by activation of TERT expression and telomerase activity, which is particularly evident in vascular structures, muscle fibers, and satellite cells. This finding prompted us to query whether expression of telomerase *per se* would promote angiogenesis. In agreement with the results of others showing that hTERT-expressing cells have increased angiogenic properties, (25) we found that adenoviral transduc-

tion of the hTERT gene in ischemic muscles induced angiogenesis at the level of capillaries. Notably, the arteriolar bed formation was not stimulated by TERT expression, suggesting that telomerase may be a necessary but not sufficient downstream effector of VEGF-dependent angiogenesis and/or that different mechanisms underlie the formation of the two vascular districts (34). This evidence is further supported by the observation that inhibition of telomerase by a dominant negative mutant co-delivered with VEGF abrogates this response and, moreover, negates the effect of the growth factor on capillaries, arterioles, and blood flow increase. Further, our *in vitro* experiments show that VEGF treatment induces TERT expression and telomerase activity in human endothelial cells and murine differentiated myotubes. Telomerase has been associated with the proliferative capacity, survival, and functionality of endothelial cardiomyocytes (23–26, 35–37) and, more recently, cardiac stem cells (38). Our data are consistent with a similar role of the enzyme during capillary formation in our *in vivo* and *in vitro* systems. Of note, the inhibition of the PI3K signaling pathway or nitric oxide production abrogates VEGF$_{165}$-dependent induction of telomerase. Conversely, the expression of a constitutively active phosphomimetic NOS mutant induces *TERT* promoter activity at levels similar to those of VEGF. These results, confirming that PI3K and NO synthesis play an important role in the VEGF-mediated angiogenic effect (39), provide a link between the angiogenic factor and telomerase expression and function. The observations that induction of telomerase is brought about also by aFGF or bFGF, but not in either case inhibited by 7N, support a strict correlation between the enzyme and promotion of VEGF-dependent angiogenesis while at the same time suggesting that the other angiogenic factors utilize pathways other than those regulating NO synthesis. Remarkably, recent observations indicate that TERT may induce basic bFGF expression in microvascular endothelial cells (40) or epithelial growth factor may increase TERT levels directly by up-regulating its transcription (35). Therefore, the biological function of TERT, at least in vascular cells, may be controlled by a molecular network of autoregulatory loops in which growth factor production plays an important role. Interestingly, recent evidence indicates that, in near to senescence endothelial cells, TERT response to VEGF induction is inhibited (41, 42). Remarkably, in this condition, NO production in HUVEC is also deficient (36), thus suggesting that the TERT-dependent VEGF function may be compromised in aging.

We have shown that VEGF treatment of C2C12 cells activates the *hTERT* promoter, indicating that, also in our system, the increase in *TERT* mRNA and enzymatic activity could be accounted for by a mechanism operating, at least in part, at the transcriptional level. Further studies are required to better elucidate this regulatory process. The rapid onset of the angiogenic process after expression of telomerase, detectable by day 3 *in vivo* and within 12–24 h *in vitro*, would seem to exclude a telomerase effect mediated by substantial lengthening of telomeres. Rather, we favor a model in which telomerase acts as survival factor, capping chromosome ends and thereby protecting cells from apoptosis (43). Recently, a novel extranuclear function of TERT, which protects cells modulating mitochondria calcium influx, has also been reported (44). Telomere dysfunction has also been implicated in myocardiocyte apoptosis after heart failure (26, 37, 45). Thus, it is tempting to speculate that the anti-apoptotic effects of VEGF$_{165}$ may be, at least in part, imputable to telomerase.

In conclusion, here we document for the first time a positive regulation by VEGF on telomerase activity *in vitro* and *in vivo*. Given that telomerase *per se* can induce angiogenesis, it is

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reasonable to assume that activation of telomerase is an essential requisite for the VEGF-dependent remodeling of ischemic tissue. Moreover, the detection of TERT in the activated skeletal muscle satellite cells provides evidence that this cell population is a target of VEGF during *in vivo* regeneration of damaged muscle fibers after acute ischemia. Our findings may lead to the design of novel gene therapy interventions based on the combined delivery of VEGF and TERT.

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Telomerase Mediates Vascular Endothelial Growth Factor-dependent Responsiveness in a Rat Model of Hind Limb Ischemia

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