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Induction of Tubulogenesis in Telomerase-Immortalized Human Microvascular Endothelial Cells by Glioblastoma Cells

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To facilitate the study of human endothelial cells we have used a replication defective retrovirus encoding the catalytic subunit of telomerase (hTERT) to derive populations of telomerase-immortalized human microvascular endothelial (TIME) cells. Whereas parental HMVECs became senescent on average within 35–45 population doublings (PDs), TIME cells have continued to proliferate for at least 200 PDs. TIME cells express readily detectable telomerase activity but display only a modest increase in telomere length. Karyotypic analysis reveals the cells to have a normal complement of human chromosomes with no evidence of gross genetic abnormalities. Furthermore, TIME cells retain many of the characteristics of the primary endothelial cells from which they were derived. For example, they express a panel of characteristic endothelial cell surface marker proteins such as CD31/PECAM-1 and $\alpha v\beta 3$-integrin. In addition, TIME cells express receptors for low-density lipoprotein (LDL) receptor as they are competent for receptor-mediated endocytosis of fluorescent acetylated LDL. Importantly, when plated on matrigel, TIME cells undergo tubule formation. Moreover, when cocultured in the presence of human glioma cells, but not primary human astrocytes, TIME cells are induced to form stable tubules. Detachment of TIME cells from extracellular matrix leads to a form of programmed cell death known as anoikis. Conditional activation of the protein kinase Akt (Akt:ER*) significantly inhibited the onset of TIME cell anoikis under these conditions. We believe that the ability of hTERT to immortalize primary human endothelial cells, and the fact that such cells retain the endothelial characteristics of the cells from which they were derived, will greatly facilitate the analysis of human endothelial cell biology *in vitro*.

Key Words: **endothelial cells; telomerase; tubulogenesis; Akt; apoptosis.**

INTRODUCTION

A major barrier to the study of primary human cells is the difficulty in growing long-term cultures of such cells *in vitro*. Prolonged expansion of primary human cells in culture is limited by the phenomenon of replicative senescence [1, 2]. In general, primary human cells are capable of 40–80 population doublings (PDs) *in vitro* before they cease proliferation and enter a metabolically viable but irreversibly postmitotic state [3]. Although expression of DNA tumor virus oncoproteins such as SV40 large T antigen can extend the lifespan of primary human cells in culture, it does so by neutralizing the activity of pRb and p53, essential regulators of the normal cell division cycle, genomic integrity, and apoptosis. Hence, such cells frequently display significant alterations in cell physiology, which limits their utility. Furthermore, although DNA tumor virus oncoproteins extend the cellular lifespan, cells eventually undergo cell death due to telomere attrition in a process known as crisis. Immortalized variants that emerge from crisis often exhibit substantial genomic alterations. Clearly, an alternate method for extending human cell lifespan, in which the cells retain the normal biological properties of the primary cells from which they were derived, would be a valuable tool permitting a systematic analysis of human cell biology *in vitro*.

Considerable evidence has accumulated to suggest that the process of replicative senescence *in vitro* is triggered by the shortening of telomeric repeat DNA at the ends of human chromosomes that occurs after each round of DNA replication [1, 4]. Once telomeres have shortened to a certain critical length, cells activate mechanisms that irreversibly arrest the cell cycle, leading to cellular senescence. In cells with an extended lifespan, such as stem cells and cancer cells, telomere attrition is offset by the activity of a ribonu-

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cleoprotein reverse transcriptase known as telomerase, which functions by adding telomeric repeats to the chromosome ends [5]. Remarkably, the expression of the catalytic subunit of telomerase (hTERT) in a number of primary human somatic cells is sufficient to confer an extended lifespan on the cells [6–8]. Furthermore, hTERT immortalized human fibroblasts are not transformed, have a normal karyotype, and are arrested in response to growth factor deprivation and cellular stresses [9, 10]. Hence immortalization of cells by hTERT expression may represent a new and valuable method of generating long-term cultures of human cells for *in vitro* analysis.

Here we describe the use of a replication-defective retrovirus vector encoding hTERT to immortalize human neonatal, dermal microvascular endothelial cells. Such telomerase-immortalized microvascular endothelial (TIME) cells retain many of the endothelial properties of the primary endothelial cells from which they were derived, including cell surface expression of CD31/PECAM-1 and the $\alpha v\beta 3$-integrin heterodimer. TIME cells retain the capacity for uptake of acetylated low-density lipoprotein (LDL) and for tubulogenesis. TIME cells retain a normal karyotype and do not show signs of transformation. Furthermore, given the reported importance of the PI3′-kinase–Akt pathway in cell signaling, we have used a conditional form of Akt to explore the influence of this pathway on apoptosis in TIME cells. Finally, TIME cells form tubules when cocultured in the presence of a human glioblastoma derived cell line but not in the presence of primary human astrocytes. Consequently, we believe that TIME cells may be a useful system for investigating the process of tumor cell induced endothelial cell tubulogenesis *in vitro*. In addition, we believe that hTERT immortalized human endothelial cells will allow us to explore in greater detail the role of various signal transduction pathways in the biology of human endothelial cells.

MATERIALS AND METHODS

Cells and media. Normal HMVEC were obtained from Clonetics (San Diego, CA). Cells were cultured in full medium, EGM-2MV (endothelial growth medium), supplemented with 5% fetal bovine serum (FBS) and EGF, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin, hydrocortisone, and GA-1000. Glioma cells (U251) were obtained from Dr. Russ Pieper's lab and cultured in DMEM supplemented with 10% (v/v) FBS. Human neonatal dermal fibroblasts were obtained from Clonetics and cultured in DMEM supplemented with 10% (v/v) FBS.

Retroviral infection of cells. A cDNA encoding hTERT, the catalytic subunit of telomerase (gift of Dr. R. Weinberg, Whitehead Inst.), was subcloned into the pWZLblast3 retrovirus which also encodes resistance to the drug blasticidin. Recombinant amphotropic retroviruses were generated by transient transfection of Phoenix A packaging cells as described previously [11, 12]. Primary HMVEC infected with the WZLblast3:hTERT retrovirus were selected in complete endothelial cell medium containing blasticidin (25 μg/ml) for 4 days. Drug-resistant cells were trypsinized and established as pooled cultures of endothelial cells. Cells expressing conditionally active Akt:ER* were derived by infection with an amphotropic retrovirus encoding a fusion protein consisting of an activated form of Akt fused to a modified form of the hormone binding domain of the mouse estrogen receptor α [13]. This virus also encodes resistance to the drug puromycin. Drug-resistant cells were selected with puromycin (2 μg/ml) for 4 days. A pooled population of cells expressing Akt:ER* was trypsinized and expanded and subsequently cloned by limiting dilution.

Telomeric repeat amplification protocol (TRAP) assay. Telomerase activity was assessed by a PCR-based, telomeric repeat amplification protocol (TRAP assay, Pharmingen) as previously described [14] and as outlined in the Boehringer TRAP assay kit (Boehringer Mannheim). The telomeric repeat 6-bp ladder products of the TRAP reaction were separated using a 10% (w / v) nondenaturing polyacrylamide gel and visualized using Kodak X-ray film.

Telomere size estimation by fluorescence in situ hybridization. Telomere length was assessed by quantitative fluorescence *in situ* hybridization (FISH) using a peptide nucleic acid (PNA) probe specific for the telomeric sequence labeled with Cy3 (Dako Code No. K 5326). Briefly, metaphase spreads were incubated for 2 h at room temperature with the PNA–Cy3 probe, washed, and counterstained with DAPI. Digital images of the metaphase spreads were captured for analysis of fluorescence intensity of each chromosome end. A range of five to eight metaphases per cell population were captured and analyzed. The results were pooled and compiled as a histogram.

Expression of endothelial cell surface markers. Staining for cell surface markers was performed using mouse monoclonal antibodies specific for human PECAM-1/CD31 (Dako Corp.), a microvascular endothelial cell marker, and the $\alpha v\beta 3$–integrin complex (LM609; Chemicon International). Immunostaining was detected using either a phycoerythrin- or a FITC-conjugated anti-mouse immunoglobulin antibodies. In each case cells were stained with the appropriate mAb isotype as a control.

Uptake of fluorescent acetylated LDL. Monolayers of endothelial cells that were 70–80% confluent were incubated with acetylated low-density lipoprotein (DiL-AcLDL; Molecular Probes) at a final concentration of 10 μg/ml for 4 h in the cell culture incubator. Cells were washed twice with PBS, harvested, and analyzed for uptake of fluorescent DiL-AcLDL by flow cytometry.

Matrigel tubulogenesis assay. Tubule formation by endothelial cells was assessed by using growth factor reduced matrigel matrix (Becton–Dickinson) which was liquefied overnight at 4°C. Liquefied matrigel was diluted 1:1 with serum-free medium and added to the wells of a 48-well tissue culture dish to solidify. After a 30-min incubation at 37°C, $2–5 \times 10^4$ cells were added to each well. Cells were cultured for 24 h. Tubulogenesis was monitored by direct inspection of the cells by phase contrast microscopy.

Karyotype analysis. Karyotype analysis was performed by standard techniques [15]. Briefly, metaphase spreads were prepared from cells that were treated with 100 ng/ml Colcemid for 4 h prior to staining (KaryoMAX, Gibco-BRL). Standard G-banding karyotypic analysis was performed on at least 20 metaphase spreads for each cell population.

Analysis of protein expression by Western blotting. Cells were lysed in a buffer containing 20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM EDTA, and 0.5% (w / v) SDS containing a standard mix of protease and phosphatase inhibitors [16]. The concentration of cell lysates was measured using the BCA assay kit (Pierce). A total of 50 μg of extracted cellular protein was resolved by polyacrylamide gel electrophoresis and analyzed by probing Western blots with the appropriate antisera. Antibodies specific for various human proteins were used including polyclonal phosphospecific α-ERK1/2 (P-T202/PY-204, New England Biolabs); phosphospecific α-Akt (P-S473, gift from Dr. D. Stokoe), phosphospecific α-Bad (P-S112/P-S136 New England

Biolabs), polyclonal α-ERK2 (C14, Santa Cruz Biotechnology), polyclonal α-hbER (HC-20, Santa Cruz Biotechnology), α-p21^{Cip1} (Ab-1, Calbiochem), α-p16^{Ink4a} (Ab-1 and Ab-2, NeoMarkers), and α-p27^{kip1} (Transduction Labs). Antigen:antibody complexes were detected using horseradish-peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulins (Amersham) at a dilution of 1:5000 as appropriate and visualized using the SuperSignal Ultra chemiluminescence detection kit (Pierce).

Anoikis assay. A total of 10^6 cells at a final concentration of 2.5×10^5/ml were cultured in the presence of full medium on polyhemecoated plates in a final volume of 4 ml. Cells and medium were collected at designated time points and lysed in a buffer containing 0.1% (v/v) NP40 for 20 min on ice and nuclei were collected by centrifugation. Apoptosis was assessed using the TUNEL assay as previously described [17].

Endothelial cell coculture assays. TIME cells (between 24 and 30 passages/120–150 population doublings) were seeded with U251 cells at a ratio of 10:1 (total cell counts of 10^4:10^3) or 100:1 (total cell counts of 10^6:10^4) on glass coverslips (Corning) in six-well tissue culture plates. Cells were maintained in endothelial cell medium supplemented with growth factors which was changed every 48 h. At different times after initiation of the experiment cells were fixed with ice-cold methanol for 60 s and stored in 70% (v/v) ethanol at 4°C. Tubules in cocultures were stained with FITC-labeled Ulex Europaeus agglutinin lectin I (UEA-1; Vector Laboratories) which is specific for microvascular endothelial cells. Nuclei were counterstained with propidium iodide according to the manufacturer's instructions (Pharmingen). Images were obtained using a Zeiss LSM 510 confocal microscope equipped with the META spectral emission detection system. The 488-nm line from an argon laser was used for excitation of both FITC and PI fluorochromes. Obtained images were collected at $1024 \times 1024 \times 40$ voxel resolution. Z-series stacks were acquired at 0.95-μm steps.

RESULTS

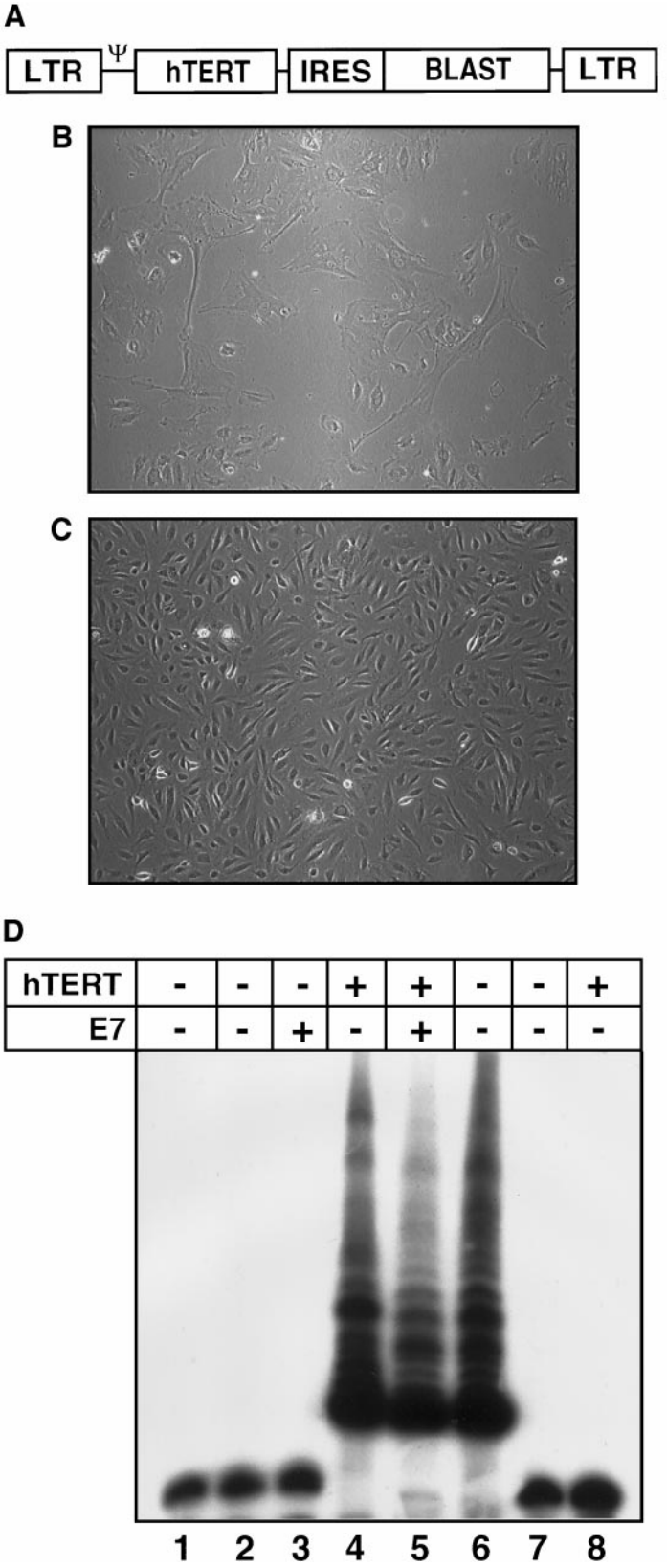
Expression of hTERT in Human Dermal Microvascular Endothelial Cells

To express hTERT in human cells, we constructed a replication-defective retrovirus expression vector encoding hTERT and resistance to blasticidin (pWZL-blast3:hTERT, Fig. 1A). In this vector both hTERT and resistance to blasticidin (Blast) are expressed from a single bicistronic mRNA with the translation of the blasticidin resistance gene promoted by the internal ribosome entry site from encephalomyocarditis virus [18]. Amphotropic retrovirus stocks were produced as described under Materials and Methods and used to infect primary human microvascular endothelial cells (HMVECs, Clonetics) at 10–15 population doublings. Blasticidin-resistant cells were selected and then trypsinized and expanded as a pooled culture. Whereas the parental HMVECs ceased proliferation after approximately 42 PDs, HMVECs expressing hTERT have continued to proliferate for up to 200 PDs. Control HMVECs that had undergone senescence displayed a characteristic flattened cell morphology (Fig. 1B) and stained positive for senescence-associated β-galactosidase (SA-β-gal) activity (data not shown) [19]. In contrast, TIME cells retained the morphology of the primary HMVECs from which they were derived (Fig. 1C) and did not express SA-β-gal. This experiment was conducted on six separate occasions and each time immortalized endothelial cells were readily isolated following infection with the hTERT encoding retrovirus. By contrast, no immortalized endothelial cells were derived from uninfected HMVECs. Furthermore, as a control in these experiments, HMVECs were infected with a retrovirus expressing the E7 oncoprotein of human papilloma virus 16 (HPV-16). Virus infected cells were expanded in mass culture and continuously propagated. In contrast to the hTERT expressing cells, E7 expressing cells underwent senescence displaying similar characteristics as uninfected senescent HMVECs (data not shown). To test whether E7 potentiated the ability of hTERT to immortalize HMVECs, the E7 expressing cells described above were infected with the hTERT encoding retrovirus and blasticidin-infected cells were selected. In this experiment, the frequency of immortalized HMVECs was similar to cells infected with hTERT alone, suggesting that E7 is neither required nor does it potentiate the ability of hTERT to immortalize these cells (data not shown).

Telomerase Activity, Telomere Maintenance, and Karyotype of TIME Cells

To assay telomerase activity in the various cell populations, cell extracts were prepared from parental HMVECs, TIME cells, and HMVECs expressing HPV-16 E7. Telomerase activity was measured using a standard telomere repeat (TRAP) assay [20]. As a positive control, we utilized extracts derived from human embryonic kidney 293 cells in which telomerase activity was readily detected (Fig. 1D, lane 6). Telomerase activity was not detected in primary HMVECs at either 7 or 28 PDs (lanes 1 and 2). Furthermore, telomerase activity was not detected in HMVECs engineered to express the HPV-16 E7 oncoprotein (lane 3). In contrast, telomerase activity was readily detected in TIME cells as well as in HMVECs expressing both hTERT and E7 (lanes 4 and 5). As expected, telomerase activity was not detected in the buffer control (lane 7) or in heat-inactivated lysates derived from the TIME cells (lane 8). These data indicate that telomerase activity is only expressed in the HMVEC cell populations that have been transduced with the hTERT encoding retrovirus.

The ability of telomerase to prevent telomere attrition is linked to its ability to synthesize telomeric repeat DNA or to cap the telomeric ends of human chromosomes [2, 21]. The latter activity would protect the cells from senescence without an obvious increase in telomeric DNA. To determine whether the expression of hTERT led to either maintenance or extension of telomere length in TIME cells, we assessed the relative telomere length by FISH using a PNA probe as de-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scribed under Materials and Methods. Metaphase spreads of TIME cells were prepared and hybridized with a PNA probe that recognizes telomeric repeat DNA sequences (TTAGGG) but not subtelomeric DNA [22]. The fluorescence intensity of hybridization detected at the ends of each chromosome is a direct measure of telomere length [22, 23]. Metaphase spreads of parental HMVECs at 20 PDs were compared to similar spreads of TIME cells at 60 PDs. Both parental HMVECs and TIME cells displayed considerable heterogeneity in the length of individual telomeres (Figs. 1E and 1F). However, the mean telomere length in TIME cells was measured at 935 fluorescence units compared to 648 fluorescence units in parental HMVECs. In addition, the median telomere length in TIME cells was 1133 fluorescence units compared to 784 fluorescence units in parental HMVECs. These data suggest that hTERT expression promotes telomere maintenance in TIME cells. However, they also suggest that, despite readily detectable telomerase activity, there is not a substantive increase in telomere length in TIME cells compared to HMVECs.

To determine whether introduction of hTERT had a gross effect on genomic stability, TIME cells were subjected to karyotypic analysis by Giemsa staining [15]. A total of 10 metaphase spreads were examined. Each of them displayed a normal 46XY karyotype with no evidence of gross chromosome translocations or other genomic abnormalities (data not shown). Hence, hTERT expression did not have a major effect on genomic stability in TIME cells.

To determine whether hTERT expression was sufficient for oncogenic transformation of human endothelial cells, TIME cells were tested for anchorage-independent proliferation in agarose. As a control, NIH 3T3 cells transformed by v-Ha-Ras were also tested in parallel. Whereas Ras-transformed cells formed colonies in agarose with very high efficiency, TIME cells did not form colonies even when plated at high cell density. Furthermore, when injected into nude mice TIME cells did not form tumors (data not shown). Hence it appears that hTERT expression and immortalization are insufficient for oncogenic transformation of endothelial cells [24]. These data are consistent with the fact that hTERT expression is insufficient to promote oncogenic transformation of primary human astrocytes, fibroblasts, or mammary epithelial cells [25–27].

Phenotypic Characterization of TIME Cells

Immortalization of primary human cells by introduction of genes, such as SV40 large T antigen or the E6/E7 oncoproteins of HPV-16, can result in profound alterations in genomic stability and cell behavior [28]. To determine whether hTERT expression and subsequent immortalization led to alterations in the endothelial properties of TIME cells, we performed a number of tests of the endothelial phenotypes displayed by TIME cells.

Microvascular endothelial cells exhibit cell surface expression of platelet endothelial cell adhesion molecule (PECAM-1/CD31), a characteristic endothelial cell surface marker [29]. Furthermore, endothelial cells express the heterodimeric cell surface $\alpha v\beta 3$-integrin, which is reported to be essential for angiogenesis and tumor neovascularization [30]. Cell staining and flow cytometry analysis of parental HMVECs and TIME cells revealed similar levels of expression of both CD31 and $\alpha v\beta 3$-integrin (Figs. 2A–2D), as well as a number of integrin subunits such as $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 3$, and $\beta 4$ (data not shown). These data indicated that hTERT expression had not led to gross alterations in integrin receptors for extracellular matrix (ECM) adhesion. Moreover, in separate experiments, Lagunoff *et al.* have demonstrated cell surface expression of VEGF-R1 (Flk-1) and VEGF-R2 (Flt-1), which are also characteristic endothelial cell surface markers [24].

Receptor-Mediated Endocytosis and Tubulogenesis by TIME Cells

To assess whether TIME cells retained normal endothelial cell function, two endothelial-cell-specific functional assays were performed. Endothelial cells express low-density lipoprotein receptors that bind and internalize acetylated human low-density lipoprotein by receptor-mediated endocytosis [31, 32]. Primary HMVECs and TIME cells were incubated with a fluorescent form of acetylated LDL (DiL-AcLDL) and receptor-mediated endocytosis of the protein was assessed by flow cytometry as described under Materials and Methods. Both primary HMVECs and TIME cells readily took up DiL-AcLDL such that 100% of the cells were fluorescent after 4 h of incubation (Figs. 2E and 2F). Inspection of the cells by fluorescence microscopy indicated that the fluorescent form of acetylated LDL was inside the cell and not simply associated with the

FIG. 1. Isolation and characterization of TIME cells. Schematic of the retroviral vector encoding hTERT and resistance to blasticidin (A). Morphology of parental HMVECs at passage 8 (B) and TIME cells at passage 15 (C). Telomerase activity in cell extracts was measured using the TRAP assay (D). Lanes 1 and 2: HMVEC cells at passages 1 and 7, respectively; lane 3: HMVECs expressing E7; lane 4: TIME cells; lane 5: TIME cells expressing E7; lane 6: 293 cells (positive control); lane 7: PBS only (negative control); lane 8: heat-treated TIME cell extract (negative control). Telomere-PNA-FISH assay was performed on metaphase spreads to estimate telomere length in cells. The frequency of fluorescence intensity values was plotted for the parental HMVEC (E) and TIME cells (F).

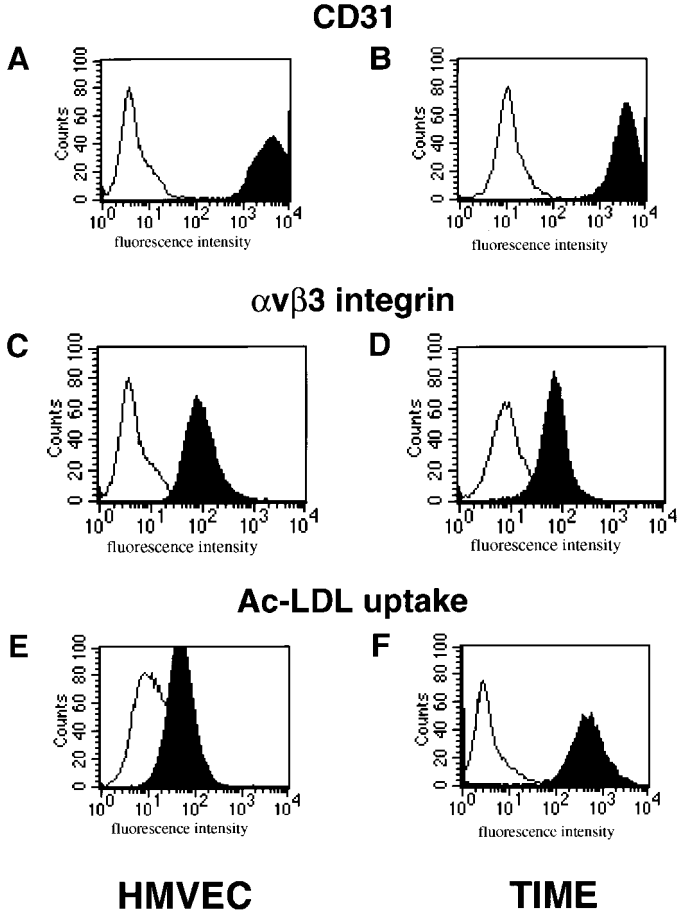

FIG. 2. Expression of endothelial-cell-specific markers and functional assay by TIME cells. Parental HMVECs and TIME cells were immunostained for cell surface expression of PECAM1/CD31 (CD31, A and B) or $\alpha v\beta 3$-integrin (C and D) as described under Materials and Methods. The uptake of fluorescent DiL-AcLDL was assessed by incubating parental HMVEC (E) or TIME cells (F) with DiL-AcLDL for 4 h. Uptake of LDL (black shaded) was compared to that of cells incubated with medium alone using flow cytometry.

outside of the cell. Hence, hTERT expression did not alter the ability of TIME cells to display receptor-mediated endocytosis of fluorescent LDL.

A striking feature of endothelial cells is their capacity to undergo tubulogenesis under appropriate conditions *in vitro,* such as when plated on matrigel, a reconstituted basement membrane extract [33]. To compare the ability of HMVECs and TIME cells to form tubules *in vitro,* the cells were plated onto matrigel and the extent of tubulogenesis was assessed by microscopic examination of the cells at different times after plating. Tubulogenesis was detected in primary HMVECs and TIME cells within 6 h after plating and a substantial tubule network had formed within 24 h (Figs. 3A and 3B). The tubule network remained stable for an additional 20 h but had disintegrated by 48–72 h after plating. We could detect no significant difference between the ability of primary HMVECs and TIME cells to form tubules when plated on matrigel. When this assay is conducted in the presence of FBS but in the absence of VEGF, bFGF, and IGF-1, the cells remain competent to form tubules within 4 h but, under these conditions, the tubules rapidly disintegrate within 12 h after initial plating (data not shown).

Next we investigated the ability of TIME cells to form tubules in a tumor cell coculture assay. TIME cells display a typical endothelial cell cobblestone morphology when grown as monolayers on tissue culture dishes in the presence of the full medium (Fig. 1C). In addition, the cells display contact inhibition when they reach confluency. Under these conditions TIME cells are never observed to form tubules. Glioblastoma multiforme tumors are highly vascularized (reviewed in [34]), hence we decided to investigate whether coculture of TIME cells with a human glioblastoma-derived cell line could promote endothelial cell tubulogenesis in the absence of collagen or matrigel. TIME cells were cocultured at a ratio of 10:1 with the glioblastoma cell line U251 in the presence of endothelial cell medium supplemented with angiogenic growth and survival factors (full medium). Within 7 days, the cocultured cells had formed a confluent monolayer. Under these conditions U251 cells proliferated well and showed no alterations in growth rate when maintained in the presence of the endothelial cell medium. Two days after cells reached confluency we observed the formation of tubule-like structures in areas of the dish (Figs. 3C and 3D). Such tubule-like structures were only observed in cocultures of TIME and U251 cells and never in the normal cultures of these cells. The tubule structures were most apparent in areas where the two cell types were growing in proximity to one another. By day 12, these tubule-like structures were extensive and covered large areas of the tissue culture dish (Fig. 3C). To investigate whether the ratio of TIME:U251 cells was important for TIME cell tubulogenesis, these experiments were repeated at a higher total cell number and at a 100:1 ratio of TIME:U251 cells. Surprisingly, although the cell cultures were confluent within 24 h, there was no evidence of tubulogenesis within this time period. However, an extensive network of tubule-like structures formed by 5–7 days after plating and this network of tubules was maintained for a further 7 days (Fig. 3D). Under these conditions the tubule network that developed was more extensive than that observed in the lower ratios. Tubule formation was not observed when TIME or U251 cells were plated alone no matter what the cell density, suggesting that the U251 cells are promoting TIME cell tubulogenesis under these circumstances.

To test whether the ability of U251 cells to promote TIME cell tubulogenesis was also a property of normal human cells, TIME cells were cocultured under the

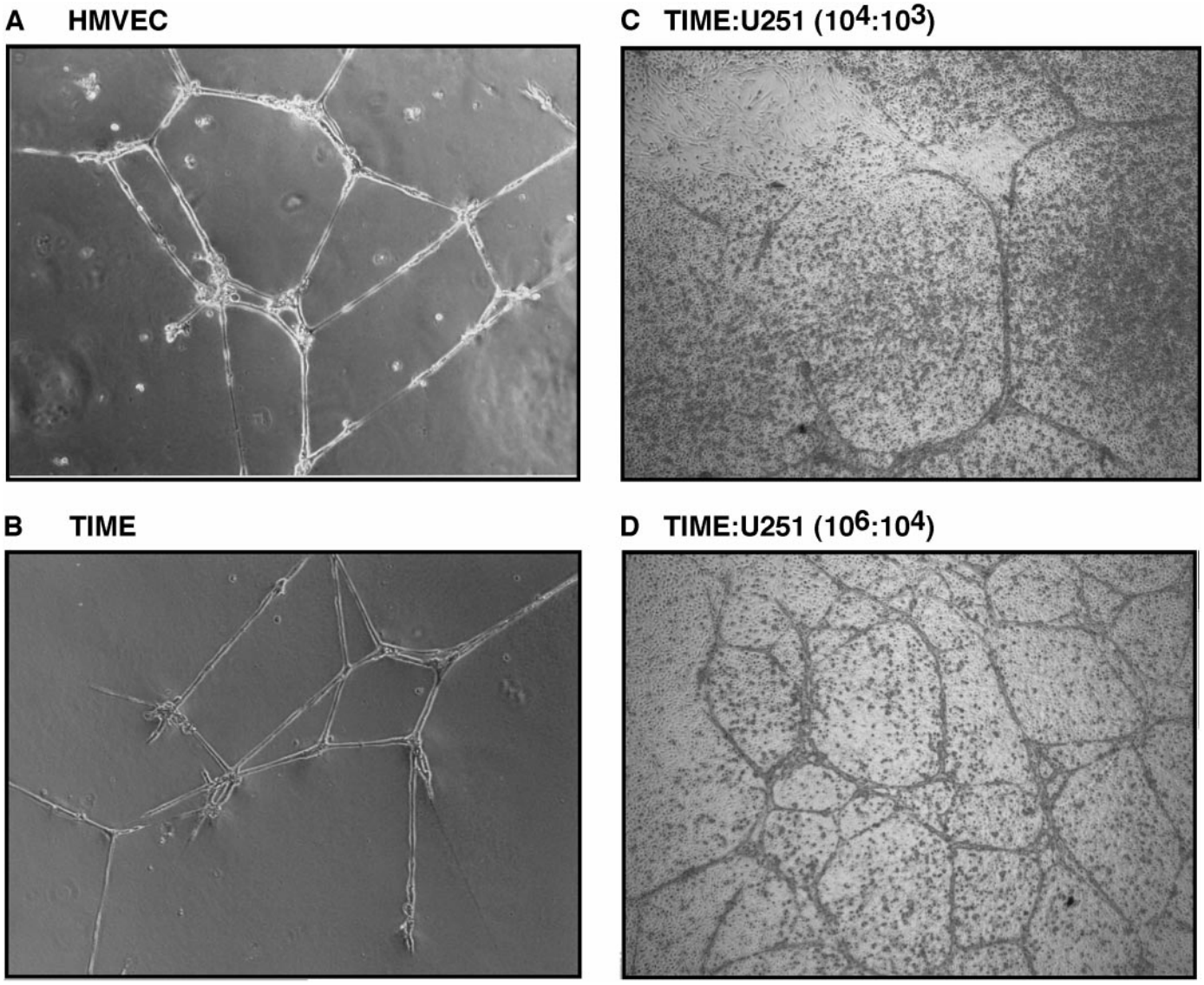

FIG. 3. Tubule formation exhibited by TIME cells. Parental HMVECs (A) or TIME cells (B) were seeded at concentration of 1×10^5/ml on matrigel in the presence of full endothelial cell growth medium. Tubule formation was monitored over 24 h. Tubule formation was examined in cocultures of TIME cells and U251 glioblastoma cells. TIME cells were cocultured with U251 cells at a ratio of 10:1 (10^4:10^3, C) or 100:1 (10^6:10^4, D) in the presence of endothelial cell medium. Medium was changed every 48 h over a time period of 7–14 days.

same conditions with primary normal human astrocytes, the cells from which glioblastoma multiforme is believed to originate. In addition, we also cocultured TIME cells with telomerase immortalized human dermal fibroblasts (NHDF-hT) or telomerase immortalized renal tubule proximal epithelial cells (RTPEC-hT) (data not shown). At no time after plating did we observe the appearance of tubules in these coculture experiments, suggesting that, at least in these experiments, nontransformed human cells were unable to promote TIME cell tubulogenesis. The lack of tubules in these experiments was not due to the loss of the TIME cells as they were readily detected by immunostaining (see below).

To explore the ability of U251 cells to promote TIME cell tubulogenesis *in vitro* and to confirm that the tubules consisted of TIME cells, cocultures of U251 and TIME cells were plated on glass coverslips and cultured until tubules formed. The cells were then fixed and immunostained for endothelial-specific cell surface markers such as von Willebrand factor (vWF) or PECAM-1/CD31. Expression of both vWF (data not shown) and CD31 (Fig. 4A) was readily detected on the tubules but not on the surrounding U251 cells. Fur-

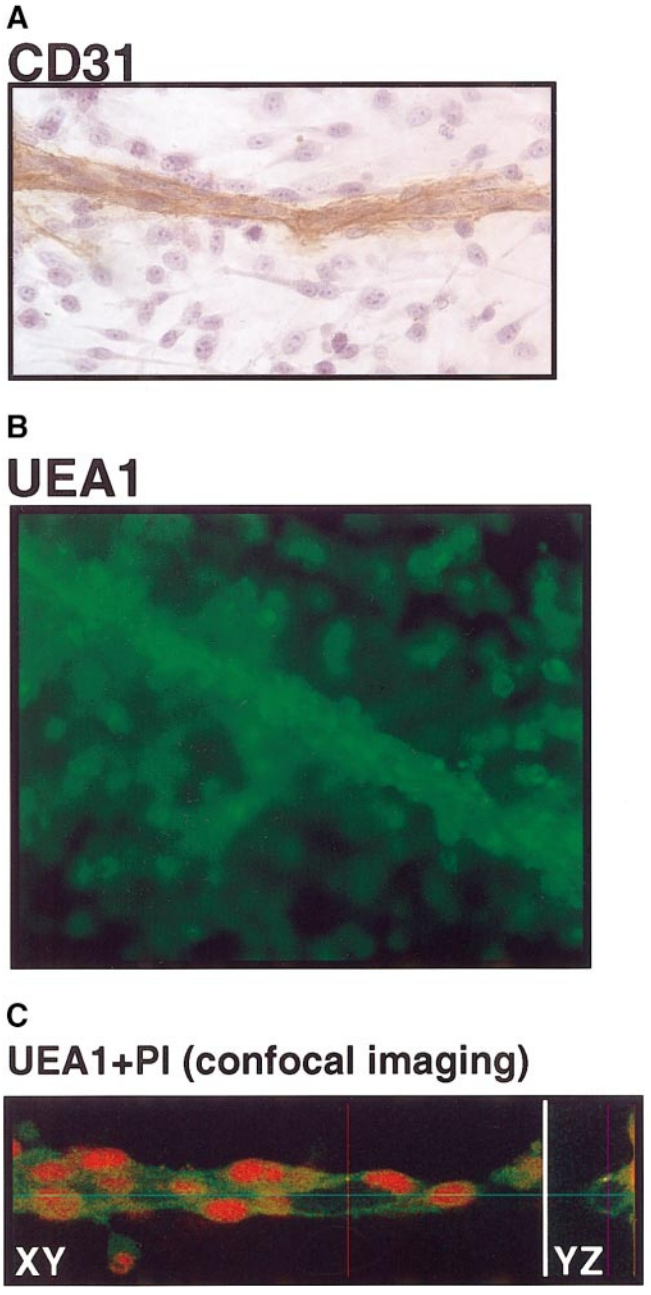

FIG. 4. Tumor-cell-induced tubulogenesis in TIME cells. TIME cells were cocultured with U251 cells on glass coverslips in a six-well plate for a period of 7–12 days prior to methanol fixation. Cells were stained with an antibody specific against CD31 (A). Staining was detected using diaminobenzidine detection and cells were counterstained with hematoxylin–eosin. Endothelial cell tubules were stained with FITC-labeled Ulex Europaeus agglutinin 1 (UEA-1), a lectin that specifically stains microvascular endothelial cells (B). Confocal microscopy was performed on cells stained with FITC-labeled UEA-1. Nuclei were labeled with propidium iodide (C). The YX section displays an longitudinal view of the tubule at 63× magnification. The YZ section is a cross section of the tubule structure.

thermore, in cocultures of TIME and NHDF-hT cells where tubulogenesis was not observed, we were readily able to detect cells expressing CD31 or vWF, indicating that the lack of tubule formation was not due to loss of the TIME cells from the cocultures. In addition, when similar TIME:U251 cocultures were exposed to fluorescent acetylated-AcLDL for a period of 4 h, granules of acetylated-AcLDL were observed within the cells forming the tubules and not within the surrounding U251 cells (data not shown). These data are consistent with the ability of TIME cells to take up DiL-AcLDL by receptor-mediated endocytosis. Finally, when similar U251:TIME cell cocultures were stained with fluorescent labeled Ulex Europaeus agglutinin 1, a lectin that selectively stains microvascular endothelial cells, only cells found in tubules, and not the surrounding U251 cells, were stained (Fig. 4B). Indeed, adjusting the plane of focus at 40× magnification suggested that the TIME cell tubules appeared to be sitting on top of a monolayer of U251 tumor cells. To confirm that the tubule structures did in fact contain a lumen, we employed scanning confocal microscopy to optically section the tubules. Figure 4C displays the compiled z-series of a tubule stained with FITC-labeled UEA-1 (green), with nuclei counterstained using propidium iodide (PI, red). The mid-XY section illustrates the 3-dimensional view of a tubule, whereas the YZ section illustrates a cross section, in which the tubule structure contains a clearly discernable lumen. Cumulatively, the data strongly suggest that in response to coculture with human U251 glioblastoma cells, TIME cells are undergoing the process of tubulogenesis. Moreover, we do not observe a similar effect when TIME cells are cultured with nontransformed human cells.

Tubulogenesis occurring within cocultures may be influenced by soluble angiogenic factors or may rely on cellular interactions between the different cells. In order to address this question, monolayers of TIME cells were cultured in the absence or presence of conditioned medium collected from either cocultures of TIME or U251 cells or from monolayers of U251 cells alone. In these experiments we did not observe TIME cell tubulogenesis up to 14 days after the addition of conditioned medium (data not shown). These data indicate that direct interactions between the TIME and the U251 cells may be required for tubule formation in these coculture experiments.

Glioma-derived cell lines have been shown to secrete VEGF and bFGF [35–37]. To investigate whether exogenous growth factors present in the full medium were required in the cocultures for TIME cell tubulogenesis, both VEGF and bFGF were omitted from the cell culture medium used for the cocultures once the cells had reached confluency at day 4–5. Under these conditions we observed no reduction in the extent of tubulogenesis observed compared to cultures in which VEGF and bFGF were maintained. This experiment was next repeated under conditions where we omitted

all growth factors except FBS. If the cocultures were allowed to progress to the point where tubules were detected, the removal of all growth factors except FBS had little or no effect on the preformed tubules. However, if all growth factors except FBS were removed from the cocultures prior to tubule formation, the cells entirely failed to form tubules at any subsequent time analyzed. Indeed, under these conditions we could readily detect areas of the culture dish containing dead and dying cells. These data suggest that in these coculture experiments, there is a critical period in which the presence of angiogenic growth and survival factors is required for TIME cell tubulogenesis. However, it seems that once tubules have formed they are relatively stable following removal of most of the factors.

Biological Effects of Akt Activation in TIME Cells

Binding of bFGF or VEGF to their cognate protein tyrosine kinase receptors leads to the activation of a number of intracellular signal transduction pathways. A prominent effector of growth factor receptors is the phosphatidylinositol (PI) 3′-kinase, which generates a series of PI3′-lipids which are phosphorylated on the 3′ position of the inositol ring. PI3′-lipids are important second messengers within the cell [38]. Binding of PI3′-lipids to a number of proteins influences their function. A prominent target of PI3′-kinase is the protein kinase Akt, which is activated upon binding of certain PI3′-lipids and phosphorylation by a second PI3′-lipid-dependent protein kinase, PDK-1 [38]. Once activated, Akt is reported to phosphorylate proteins involved in the control of transcription, the cell division cycle, and apoptosis. For example, direct phosphorylation of the BH3-only protein Bad, the cysteine protease Caspase 9, and transcription factors of the Forkhead and NF-kB family have been implicated in the ability of Akt to promote cell proliferation [39–48].

The ability of TIME cells to proliferate continuously in culture provides an opportunity to genetically manipulate the cells to allow an exploration of the effects of signal pathway activation on the cells' biological properties. To assess the role of Akt regulated signaling pathways in human endothelial cell biology *in vitro,* we used retrovirus-mediated gene transfer to derive TIME cells expressing a conditionally active form of Akt (Akt:ER*). This conditionally active protein kinase was generated by fusing DNA sequences encoding a constitutively active form of Akt to sequences encoding a modified form of the hormone binding domain of the mouse estrogen receptor α [49, 50]. Addition of estrogen analogs such as 4-hydroxytamoxifen (4-HT) to cells expressing Akt:ER* leads to rapid activation of the fusion protein with concomitant activation of downstream signaling pathways. Such conditional protein kinases have been most effective in deciphering the effects of signal pathway activation on patterns of gene expression and their subsequent effects on cell function [13, 51, 67].

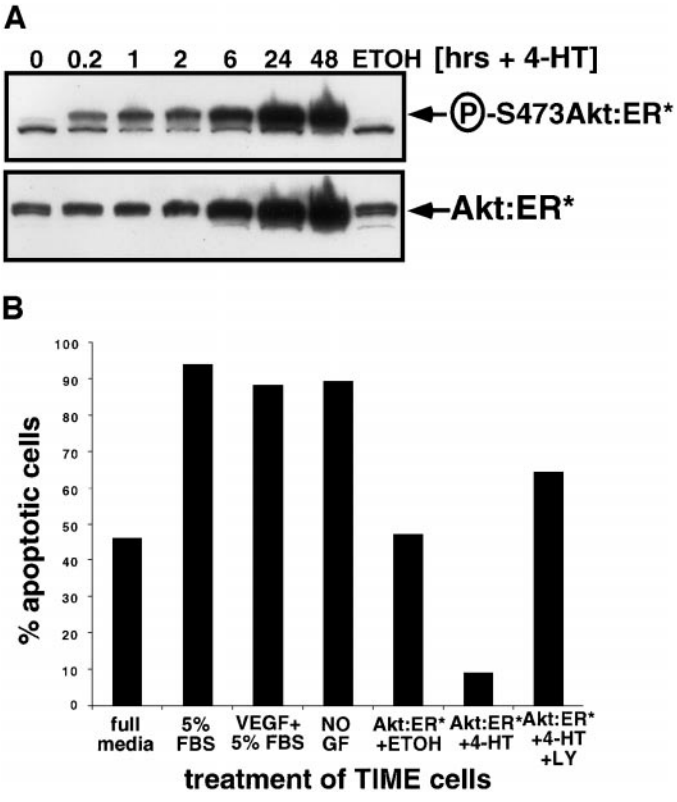

FIG. 5. Suppression of apoptosis by conditionally active Akt. TIME cells expressing conditionally active Akt:ER* were derived by retrovirus infection. Activation of Akt:ER* was assessed by Western blotting of cell extracts with a phospho-specific antiserum that only recognizes Akt:ER* when phosphorylated on the serine 473 equivalent position in Akt:ER* (P-S473, A). Expression of the Akt:ER* fusion protein was detected by Western blotting of cell extracts with an antiserum that recognizes the hormone binding domain of the mouse estrogen receptor α. Detachment-induced apoptosis (anoikis) was assessed in TIME cells cultured in suspension in polyhemecoated plates for 16 h under different conditions. Cell nuclei were collected at various time points and analyzed for apoptosis by TUNEL assay. TIME cell apoptosis was assessed in: (1) full medium; (2) medium containing only 5% (v/v) FBS; (3) medium containing VEGF + 5% (v/v) FBS; (4) medium depleted of all growth factors; (5) full medium −Akt:ER* activation; (6) full medium +Akt:ER* activation; (7) full medium +Akt:ER* activation + LY294,002, a PI3′-kinase inhibitor (B).

Consistent with previous results, addition of 4-HT to TIME cells expressing Akt:ER* led to rapid phosphorylation of serine 473 (P-S473) of Akt:ER* which was detected within 10 min after 4-HT addition and increased over the course of the next 48 h (Fig. 5A). In order to detect the overall expression of the Akt:ER* fusion protein, a Western blot was probed with a polyclonal antisera that recognizes the hormone binding domain of the mouse estrogen receptors. As previously reported, in addition to the rapid activation of the preex-

isting Akt:ER* protein, addition of 4-HT also led to elevated expression of the Akt:ER* fusion proteins at late times (≥24 h) after hormone addition. These data indicate that we can selectively activate the Akt signaling pathway in TIME cells in the same way that we have described for other kinase:ER fusion proteins in mouse, rat, canine, and human cell lines [13, 16, 52, 53, 67].

Endothelial cells require signals from the extracellular matrix to promote the cell division cycle and to prevent apoptosis. Detachment of cells from the ECM results in a form of apoptosis, known as anoikis [54]. Since Akt has been implicated in the regulation of apoptosis in endothelial cells, we investigated whether activation of Akt could influence anoikis in TIME cells. TIME cells were trypsinized and retained in suspension in polyhemecoated plates that prevent cell attachment. At different times after detachment, cell nuclei were collected and the presence of apoptotic cells was assessed using the TUNEL assay [55]. Cell nuclei were counter stained with propidium iodide and the number of apoptotic cells was calculated as the percentage of TUNEL- or PI-positive cells in the culture. In the presence of endothelial cell medium supplemented with various angiogenic growth factors (full medium), fewer than 20% of TIME cells were apoptotic after 8 h in suspension. However, after 16 h the percentage of apoptotic cells increased to 40–50% (Fig. 5B). By 24 h, the vast majority of TIME cells had died by apoptosis (data 2not shown). TIME cells maintained in suspension in the absence of angiogenic growth factors were more susceptible to apoptosis such that 90–100% of cells were apoptotic after 16 h in suspension in serum-free medium or basal medium supplemented with 5% (v/v) FBS in the absence or presence of VEGF (Fig. 5B). To determine whether Akt could influence the onset of apoptosis in endothelial cells, TIME cells expressing Akt:ER* were cultured in suspension in the absence or presence of 4-HT. In these experiments Akt:ER* was activated by 4-HT addition 24 h prior to trypsinization. In the absence of Akt:ER* activation the cells died at the same rate as the parental TIME cells, indicating that the cells were not intrinsically resistant to apoptosis as a result of retrovirus integration or some other adventitious event. Cells in which Akt:ER* was activated displayed a striking reduction in the number of apoptotic cells from 46 (control) to 8% after 16 h of culture in suspension (Fig. 5B). The anti-apoptotic effects of Akt:ER* activation were completely reversed by treatment of cells with the PI3′-kinase inhibitor LY294002, which has been shown to prevent the activation of Akt:ER* [13, 56]. These data suggest that Akt:ER* activation elicits a potent anti-apoptotic effect in TIME cells.

As described above, withdrawal of growth factors prior to tubule formation in the TIME:U251 coculture experiments results in death of the TIME cells and a concomitant failure of the cells to undergo tubulogenesis. To determine whether Akt activation might suppress apoptosis and therefore promote tubule formation in cocultures of TIME and U251 cells that were growth factor deprived on day 4 (prior to tubule formation), we used the Akt:ER* expressing cells in these coculture experiments. TIME:U251 cocultures were prepared and then growth factor deprived in the absence or presence of 4-HT to activate Akt:ER*. Surprisingly, although the TIME cells in these cocultures appeared to survive for longer periods of time compared to the control, we did not observe a restoration of tubule formation in these experiments. These results suggest that, although Akt can suppress TIME cell apoptosis following cell detachment or growth factor withdrawal, Akt activation is insufficient to promote tubulogenesis in the absence of exogenous angiogenic factors.

DISCUSSION

By expression of hTERT, the catalytic subunit of telomerase, we have successfully isolated immortalized populations of human microvascular endothelial cells. TIME cells appear to be capable of indefinite cell proliferation *in vitro* and also appear to have retained many of the phenotypic characteristics of the primary endothelial cells from which they were derived including a normal cell morphology and the capacity to form tubules *in vitro*. In addition, the cells show no gross chromosomal abnormalities and are not oncogenically transformed, consistent with previous observations that hTERT expression is insufficient to transform primary human cells. In separate studies, the endothelial nature of TIME cells has been further confirmed by the demonstration that they are competent to support infection by the human Herpes virus KSHV/HHV-8, a virus with a demonstrated tropism for endothelial cells [24]. These data are consistent with the work of others who have demonstrated that hTERT immortalized fibroblasts and retinal pigment epithelial cells retain normal cell characteristics and do not display the features of transformed cells [9, 10]. The characteristics of TIME cells and previously described hTERT immortalized human cells lead us to believe that this strategy will greatly facilitate our ability to explore human cell biology *in vitro*.

Others have sought previously to immortalize primary human cells by expression of the DNA tumor virus oncoproteins of HPV-16 (E6 and E7), SV40 (large T antigen), or adenovirus (E1A). Indeed, in one study, immortalized human umbilical vein endothelial cells were immortalized by expression of E6 and E7 and were shown to retain phenotypic and functional characteristics of the parental endothelial cells [57]. However, karyotypic analysis revealed that these cells had sustained substantive genomic alterations. Since DNA tumor virus oncoproteins have pleiotropic effects on cell physiology and frequently inhibit or abrogate the function of p53 and/or pRb and its family members, it is not surprising that primary

human cells immortalized in this way are prone to a variety of genomic alterations.

Immortalization of human endothelial cells of the umbilical vein, saphenous vein, aorta, coronary artery, and dermal origin by retroviral expression of hTERT has recently been reported by others [58]. Like TIME cells, these cells exhibited functional characteristics of the primary endothelial cells from which they were derived. However, the authors noted aneuploidy and karyotypic abnormalities in some of the immortalized populations derived in their study. However, it remains unclear whether the genetic alterations occurred as a consequence of hTERT expression and immortalization, since genetic abnormalities were also detected in the primary endothelial cells from which the immortalized variants were derived. Since we detected no obvious genetic abnormalities in TIME cells, it is clear that hTERT expression does not invariably lead to genomic damage in primary human endothelial cells. Clearly, cells that retain the normal expression/function of genes involved in a variety of stress and damage responses such as p53, pRb, $p14^{ARF}$, and $p16^{Ink4A}$ will be useful for studying the *in vitro* response of endothelial cells to the effects of both pro- and anti-angiogenic agents as well as a variety of other agents that may influence the endothelial cell division cycle, migration, tubulogenesis, or apoptosis. Indeed, "telomerized" human neonatal dermal microvascular endothelial cells have been demonstrated to form functional anastamoses with mouse vasculature when implanted into immunocompromised (SCID) mice [59]. Such observations open the possibility of testing the effects of pro- and anti-angiogenic agents on human endothelial cells in an *in vivo* setting.

To date, hTERT expression has been reported to immortalize a large number of human cell types including cells of mesenchymal, epithelial, and hematopoietic origin [6–8, 25]. However, it has been reported that the immortalization of human keratinocytes and mammary epithelial cells requires the collaboration of hTERT and the E7 oncoprotein of HPV-16. By contrast, recent evidence suggests that the apparent requirement for additional genetic elements such as E7 in these experiments is a reflection of inadequate cell culture conditions as opposed to an intrinsic inability of hTERT to immortalize primary human cells [60]. Certainly, in our hands, the presence of E7 was neither required nor did it facilitate the immortalization of endothelial cells by hTERT expression.

An interesting feature of the ability of hTERT to immortalize endothelial cells is the fact that, despite the ready detection of telomerase activity in hTERT expressing cells, there was only a modest increase in telomere length at the ends of the chromosomes. It is possible that telomere length maintenance and not extension is important in extending cell lifespan *in vitro*. Indeed, Blackburn and colleagues have suggested that telomerase may physically protect the ends of human chromosomes from recognition as damaged DNA by physical capping of the chromosome end [2]. The proposed telomere capping activity of telomerase prevents the cells from inducing a senescence checkpoint and subsequent irreversible cell cycle arrest that is the key feature of replicative senescence *in vitro*.

In this paper, we describe an *in vitro* assay that allows the investigation of cellular interactions involved in endothelial cell tubulogenesis. In this assay a human glioblastoma cell line, but not primary human astrocytes, elicited TIME cell tubulogenesis *in vitro*. The apparent tumor cell specificity of this may now be explored using additional tumor cell lines as well as primary human astrocytes that have been rendered tumorigenic by the expression of a variety of oncogenes [26, 27]. However, it is clear that the ability of TIME cells to form tubules in response to coculture with U251 cells is not simply due to the release of a autocrine/paracrine endothelial growth and differentiation factor since conditioned medium from U251 cells or TIME:U251 cocultures was without effect when applied to monolayers of TIME cells. These data suggest that endothelial growth factors work in concert with intimate cell–cell contact to promote TIME cell tubulogenesis. Indeed, the secretion of endogenous growth factors may rely on specific cellular interactions between the TIME cells and the glioma cells.

The interactions between astrocytes and endothelial cells have been explored by others using coculture techniques in an effort to understand the functional properties of the blood–brain barrier [61–63]. Under certain circumstances coculture of HUVECs with normal human fibroblasts resulted in endothelial cell tubulogenesis [61–63]. In addition, when cultured with rat C6 glioma cells, HUVECs were shown to form tubules that developed a permeability barrier with optimal high transendothelial cell electrical resistance consistent with the formation and correct function of tight junctions [64, 65].

The ability to derive long-term cultures of human endothelial cells allows the use of additional genetic manipulations to explore the role of various signal transduction pathways in endothelial cell biology. To study the effects of Akt activation on endothelial cells we have expressed a conditionally active form of the protein kinase Akt (Akt:ER*) in TIME cells. Activation of Akt suppressed detachment-induced TIME cell apoptosis, but did not initiate tubule formation in the cocultures in the absence of growth factors. This result stands in contrast to reports that constitutively active Akt can induce angiogenesis in a chick chorioallantoic membrane assay [66]. However, this was in the presence of other growth and survival factors, indicating that Akt may act in concert with growth factors to

promote angiogenesis. Although the mechanisms by which Akt suppresses apoptosis in TIME cells are not known, the ability to maintain long-term cultures of these cells will allow us to explore the mechanisms underlying such observations in more detail [67].

Considerable interest has focused on the effects of pro- and anti-angiogenic growth factors on the activity of signal transduction pathways downstream of Ras in endothelial cells. For example, mice with a homozygous deletion of B-Raf display alterations in endothelial cell apoptosis and differentiation that leads to death of the embryos of vascular hemorrhage between day E10 and 11. In addition, the Raf→MEK→ERK pathway induces the expression of $\alpha v\beta 3$-integrin expression on the surface of a number of cell types including in TIME cells, an integrin complex intimately associated with endothelial cell survival and migration [30, 52, 68]. Consequently, we are currently investigating the role of Raf→MEK→ERK pathways in endothelial cell proliferation and tubule formation in the coculture assays and comparing the effects of activation of this pathway to the effects of activated Akt.

In summary, we believe that telomerase immortalized human endothelial cells will allow us to conduct a systematic examination of the cell and molecular biology of human endothelial cells. We believe that the study of such cells will promote our understanding of endothelial cell physiology and may be a useful platform for the design and evaluation of novel pro- and anti-angiogenic therapies.

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