Normal human endothelial cells, like other somatic cells in culture, divide a limited number of times before entering a nondividing state called replicative senescence. Expression of the catalytic component of human telomerase, human telomerase reverse transcriptase (hTERT), extends the life span of human fibroblasts and retinal pigment epithelial cells beyond senescence without causing neoplastic transformation (Bodnar, A. G., Ouelle, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., and Wright, W. E. (1998) Science 279, 349–352; Jiang, X., Jimenez, G., Chang, E., Frolkis, M., Kusler, B., Sage, M., Beeche, M., Bodnar, A., Wahl, G., Tlsty, T., and Chiu, C.-P. (1999) Nat. Genet. 21, 111–114). Here, we show that both large human vessel and microvascular endothelial cells also bypass replicative senescence after introduction of hTERT. For the first time, we report that hTERT expression in these life-extended vascular cells does not affect their differentiated and functional phenotype and that these cells maintain their angiogenic potential in vitro. Furthermore, hTERT(+)-microvascular endothelial cells have normal karyotype, and hTERT(+)-endothelial cell strains do not exhibit a transformed phenotype. Relative to parental cells at senescence, hTERT-expressing endothelial cells exhibit resistance to induction of apoptosis by a variety of different conditions. Such characteristics are highly desirable for designing vascular transplantation and gene therapy delivery systems in vivo.

The integrity of endothelial cell (EC) function is compromised with advancing age and directly contributes to age-dependent impairment of angiogenesis and development of vascular diseases (1, 2). Aging of the vasculature is also associated with a number of phenotypic and hemodynamic changes commonly observed in cardiovascular disease states (3, 4). Some of these phenotypic alterations have been found to occur in ECs undergoing senescence in vitro and include increased expression of proteolytic activity (5, 6), inflammatory markers (7–9), and vasoconstrictors and reduced expression of vasodilators and vasoprotective factors (3, 10–12).

The adult endothelium in vivo is remarkably quiescent (13, 14), but ECs can be activated to proliferate, e.g. following traumatic injury, inflammation, and tumor formation or in response to physiologic cues during hair growth and ovarian cycling (15, 16). This property has allowed the in vitro cultivation and expansion of ECs from many different tissues. Like most human somatic cells, however, ECs undergo replicative senescence in vitro after a finite number of divisions, which can vary between 20 and 60 population doublings (PDLs) depending on the tissue of origin and culture conditions (17).

Efforts to extend the life of ECs have focused on ectopic expression of viral oncogenes (18–21), spontaneous transformation (22), addition of exogenous growth factors, and provision of supportive matrix components (17). One EC-specific growth factor, vascular endothelial cell growth factor has been shown to both delay the onset of human dermal microvascular EC (HDMEC) senescence (23) and block anoikis (anchorage disruption-induced apoptosis) by producing a permissive matrix that supports EC adhesion and proliferation (24). ECs will eventually undergo replicative senescence regardless of exogenous growth factors and matrix components unless they are transformed. Immortal EC lines generated by viral or spontaneous transformation, however, invariably fail to exhibit physiologic distal proliferative signaling pathways and eventually lose important differentiated EC functions.

Another way to bypass senescence has recently been achieved by ectopic expression of the human telomerase reverse transcriptase (hTERT) gene, which imparts replicative immortality to fibroblasts and retinal pigment epithelial (RPE) cells (25) without converting to a neoplastic transformed phenotype (26, 27). A recent finding that both hTERT expression and inactivation of p53 or p16 is required to immortalize primary human keratinocytes and mammary epithelial cells raises the issue of cell type specificity in hTERT-mediated immortalization (28). In this report, we show that both large and small vessel-derived ECs, like dermal fibroblasts, can be effectively immortalized by hTERT alone in the absence of malignant transformation. In addition, we show for the first time that hTERT-immortalized ECs exhibit functional and morphogenetic characteristics of parental cells. These hTERT-EC lines may also display a survival advantage beyond the hurdling of replicative senescence, as they appear to be more resistant to programmed cell death. Such characteristics may be useful in the design of vascular model systems and therapeutic strategies for treating age-related diseases of the vasculature.
FIG. 1. Telomerase activity and telomere length in human endothelial cells after hTERT expression. A, telomerase activity measured by the TRAP assay (see under “Materials and Methods”) was detected in both early (PDL 5) parental HDMECs PD5 and all hTERT-ECs from as little as 1000 cell equivalents, but was not detected in middle or late passage parental, sham-infected (LacZ) or heat-treated (HT) hTERT-HUVECs, hTERT-HAECs, and hTERT-HDMECs. B, hTERT-HDMEC telomerase activity was quantified using PCR-ELISA and showed persistent activity up to PDL 100, at levels ranging between 80 and 120% of that of the embryonic kidney tumor cell line, 293. Similar results were obtained from two other human cell strains, hTERT-HCAECs and hTERT-HSVECs. C, TRF length of DNA from hTERT-HDMECs at different PDLS showing loss of telomeric DNA from approximately 9 to 4 kbp spanning PDL 100. D, mean TRF length at the indicated PDLS for four representative hTERT-HUVEC clones showing stabilization of telomere length at approximately 2.5 kbp.

**MATERIALS AND METHODS**

**Establishment of Endothelial Cell Cultures**—Human umbilical vein endothelial cells (HUVECs) were given as a gift from Drs. Thomas Maciag (Maine Medical Center) and Susan Garfinkel (American Red Cross). Human saphenous vein endothelial cells (HSVECs) came from Vascular Endothelial Cell (VEC) Laboratories. Human aortic endothelial cells (HAECs), human coronary arterial endothelial cells (HCAECs), and part of HDMECs for making hTERT1 were purchased from Clonetics. hTERT3 was established in our laboratory by preparation of primary HDMECs from neonatal foreskin as described previously (29, 30). Growth media were from EBM-2 (or EBM) MV BulletKit (Clonetics). Our primary parental HDMECs for hTERT3 was purified with PECAM-coated beads (Sigma) and recovered with a magnetic particle concentrator (Promega). A spontaneously transformed HUVEC line, ECV304, was obtained from ATCC.

**Gene Transduction**—Gene transfer was achieved by either electroporation-based transfection or retrovirus-mediated gene transfer. For electroporation, pZeoSV-hTERT, a derivative of pZeoSV (Invitrogen) was used to transfect HUVECs (25). Two different versions of retrovirus were used to transduce hTERT genes to the ECs. pBabe-hTERT (a generous gift from Dr. Woody Wright, University of Texas Southwestern Medical Center, Dallas, TX) was used for HAECs, HCAECs, and HSVECs (31) and LZRS-hTERT was used for HDMECs (32). The expression of hTERT for both retroviral vectors was driven by Moloney murine leukemia virus 5'-long terminal repeat promoter. Viral titers determined by the infection of NIH-3T3 cells with pBabe-hTERT or LZRS vector containing enhanced green fluorescent protein reporter gene were estimated at approximately 5 × 10⁶/ml.

**Telomeric Repeat Amplification Protocol (TRAP) Assay**—Either a non-amplified conventional standard (33) or a PCR-ELISA-based assay (Roche Molecular Biochemicals) was used to measure the telomerase activity from the hTERT transgene or endogenous telomerase. The standard protocol was used for HUVECs, HAECs, HCAECs and HSVECs, whereas PCR-ELISA was used for HDMECs. For visualizing the DNA ladder with the standard protocol, 1000 or 5000 cell equivalents were analyzed. For PCR-ELISA, 2000 cell equivalents were used. The PCR-ELISA protocol was provided by the assay kit manufacturer (Roche Molecular Biochemicals).

**Telomere Length Assay**—Isolation of genomic DNA and Southern blot determination of mean terminal restriction fragment (TRF) were performed according to published procedures (34–36). Briefly, genomic DNA (3 μg) was digested with HinfI/RsaI and run on 0.8% agarose gel. The gel was transferred onto a positively charged nylon membrane, which was hybridized at 65 °C overnight. Hybridization signals were detected by chemiluminescence or radioactivity. Calculation of mean TRF length was done as described previously (37).

**RT-PCR for Telomerase Transcripts**—The primers for RT-PCR were as follows: 1) in the hTERT gene, sense, CACCTCACCCACGCGAAAA; antisense, CCAAAGAGTTTGCGACGCATGTT; and 2) at the border of hTERT and retroviral LZRS sequence, sense, TCTCTGAAAGCCAAGACGCA; antisense, GACCAACTGCTATTGCTAGCGA. Total RNA was isolated with TRIZOL (Life Technologies, Inc.). The RT-PCR was performed by a one-step RT-PCR system according to the manufacturer (Life Technologies, Inc.).

**Flow Cytometry**—The antibodies used for flow cytometry were anti-PECAM-1 (Becton Dickinson), ICAM-1 (Pharmingen), and Apo2.7 (Im-
TABLE I
Maximal replication life span of endothelial cells

| Strain   | PDLmax* | Fold increase/senescence |
|----------|---------|--------------------------|
| HUVECsa  | 50      | 1                        |
| HAECsb   | >150    | 3.0                      |
| hTERT clones | >150    | 3.0                      |
| HCAECsb  | 147     | >2.5                     |
| pBAb     | 29      | 1.1                      |
| hTERTc   | 77      | >2.7                     |
| HSVECs   | 19      | 1                        |
| HDMCsb   | 47      | >2.5                     |
| parental | 30      | 1                        |
| LacZf    | >30     | 1                        |
| hTERT1g  | 155     | >5.0                     |
| hTERT3g  | 78      | >2.5                     |

a PDLmax represents the maximal amount of population doubling attained thus far for the hTERT-containing cells. At present, the hTERT cells are still proliferating; thus, PDLmax values are considered dynamic.
b PDLmax number for hTERT-HUVEC clones represents the average of six hTERT-HUVECs.
c hTERT1 and hTERT3 were derived from two independent retroviral infections of primary neonatal HDMEC mass cultures.
dHUVECs, HAECs, HCAECs, HSVECs, and HDMCs were taken with Zeiss inverted microscope.

RESULTS
hTERT Expression Extends EC Life Span—Our previous studies showed that ectopic expression of recombinant hTERT reconstituted telomerase efficiently in both dermal fibroblasts and RPE (25). In the present study, we have expressed hTERT in HUVECs by stable transfection and in HAECs, HCAECs, HSVECs, and HDMCs by retroviral transduction. The characteristic DNA ladder of the TRAP assay indicates specific telomerase activity in a representative HUVEC-hTERT clone and transduced HAEC-hTERT and HDMEC-hTERT mass cell cultures (Fig. 1A). A quantitative PCR-ELISA-TRAP shows that telomerase activity of hTERT+ HDMECs is maintained for over 100 PDLs, and the level of telomerase activity achieved in HDMCs is comparable to that expressed by the 293 human embryonic kidney tumor cell line (Fig. 1B). By contrast, parental HDMCs expressed a low level of endogenous telomerase transiently at early PDLs, consistent with previous reports (42). Using RT-PCR, we have also confirmed the presence of hTERT RNA transcribed from the transduced retroviral vector at PDL 60 in HDMECs (data not shown).

Table I summarizes the maximal PDL number attained for each of the five hTERT(+)/EC lines. Different parental EC strains senesced at different PDLs, exhibiting typical flattened cell morphology and unresponsiveness to growth factors (Fig. 2). By contrast, all hTERT-EC lines resembled young primary ECs in their morphology and growth response, with little or no staining with SA-β-galactosidase activity. Thus, ectopic expression of hTERT in ECs extended the replicative life span of all EC strains examined to over twice that of primary ECs, technically defining these hTERT-EC lines as immortalized (43).

To examine the effects of hTERT expression on EC telomeres, we assessed telomere lengths by TRF Southern analysis (see under “Materials and Methods”). Fig. 1C shows the change in telomere length of hTERT(+)/HDMEC mass cell cultures as a function of PDL. Telomere shortening was observed up to PDL 100–120 followed by consistent stabilization
**TABLE II**

Phenotype, function, and karyotype of hTERT(+) EC cultures

Symbols used in this table are as follows: +, positive from the assay; −, negative from the assay; ND, not determined; N, normal; AB, abnormal; ↓, decrease.

| Phenotypea | HUVEC | HAEC | HCAEC | HSVEC | HDMEC |
|------------|-------|------|-------|-------|-------|
| Von Willebrand factor | + | + | + | + | + |
| PECAM-1 | + | + | + | ND | + |
| SA-β-galactosidaseb | + | + | + | ND | + |

**Function**

| Tubele | Soft agarb | pith/ICAM-1 | VCAM-1 | E-selectin | LDL uptake | Apoptosisb | Karyotype |
|--------|------------|-------------|--------|------------|-------------|------------|-----------|
| +      | +          | +           | +      | +          | +           | ND         | AB AB AB N |

a Phosphorytic expression of EC markers (e.g. Von Willebrand factor VIII and PECAM-1).
b SA-β-galactosidase activity was strongly positive in all senescent, parental ECs but was weakly present or absent in all hTERT-EC lines.

c Matrigel was used for tubule/web formation except for HDMEC, on which collagen was overlaid.
d hTERT3 formed good tubules, whereas hTERT1 did not.
e Refers to the ability of hTERT-EC lines to grow in soft agar (see under "Materials and Methods").

f Refers to the detection of appropriate pRb phosphorylation patterns via immunoblotting in response to hTERT-EC cycle arrest (see Fig. 5).
g Refers to TNF-α-induced expression of ICAM, VCAM, and E-selectin by hTERT-EC by FACS analysis.
h Refers to both decreased nuclear fragmentation and/or expression of early apoptotic markers (e.g. Apo2.7 and/or Annexin V) of hTERT-EC versus senescent, parental control EC (see Fig. 7).
i Refers to cyogenic analysis of at least 20 metaphase chromosomes for all EC types. As defined by chromosome counting, karyotypic abnormalities consisted of 40–60% aneuploidy in all parental large vessel ECs at early PDLs that reached 100% aneuploidy by late PDLs. All large vessel hTERT(+) EC lines (PDL 50–100) had nearly 100% aneuploidy.
j GTW banding analyses of the parental HUVEC culture (PDL 28), two hTERT(+) HUVEC clones (14EYZ at PDL62.8 and 21EYZ at PDL85.8), parental HAECs (PDL 31.7) and HSVECs (PDL 10.9) showed trisomy 11 or 18 with deletions, additions, and translocations of subclones within the cultures. GTW banding analyses also showed that all microvascular parental EC and HDMEC lines, hTERT1 and hTERT3, had normal karyotypes at both early and late PDLs (PDL 5–140).
k Tetraploidy was observed in hTERT1 at PDL 140; it was not apparent at PDL 50 (Fig. 6).

at approximately 3–4 kbp. Analysis in four different hTERT-HUVEC clones showed similar results, with TRF stabilization at approximately 2.5 kbp by PDL 100–140 (Fig. 1D).

**hTERT(+) EC Lines Retain EC Characteristics**—Baseline expression of von Willebrand factor and CD31(PECAM-1) and cytokine-stimulated cell surface expression of inflammatory adhesion molecules, ICAM, VCAM, and E-selectin are regarded as key markers distinguishing ECs from other cell types both in vivo and in vitro (14, 44). Similarly, binding and uptake of acetylated LDL and formation of tubule-like structures in response to matricellular signals also define important EC functions maintained in vitro by primary ECs that have not lost their differentiated phenotype (17). Therefore we have assessed the hTERT-EC lines for EC marker expression and responses to cytokines and matricellular proteins (Figs. 3 and 4 and Table II).

Morphogenic responses were evaluated by exposing HUVEC parental mass cell cultures at PDL 27 (Fig. 3A) and a representative hTERT(+) HUVEC clone at PDL 127 (Fig. 3B) to Matrigel. Both cell populations responded similarly by efficiently forming “angiogenic webs,” whereas late passage, senescent PDL 50 HUVECs (Fig. 3E) and a spontaneously transformed HUVEC line, ECV304 (Fig. 3F), did not. Similar results were obtained for both parental HDMECs and hTERT(+) HDMECs exposed to 3D type I collagen (Fig. 3, C and D), and we found that these cells also form tubule-like structures in response to Matrigel. Senescent HDMECs did not form tubules in 3D collagen. As in parental cell lines, some variability in tubule pattern formation was apparent among different hTERT-EC strains (see Table II).

Cell surface expression of PECAM-1 by unstimulated parental HUVECs and an hTERT(+) HUVEC clone at PDL 120 (Fig. 4A) showed similar 90–95% immunoreactivity. Likewise, parental HDMEC and hTERT(+) HDMEC lines showed high PECAM-1 reactivity (Fig. 4B). Expression of Von Willebrand factor and LDL uptake also showed no differences between parental and hTERT-EC lines (Table II). Basal and TNF-α-stimulated cell surface expression of ICAM-1, VCAM-1, and E-selectin were similar in both parental and hTERT-EC lines (Fig. 4, A and B, Table II). By contrast, senescent HUVECs expressed high level of ICAM-1, which was not stimulated by TNF-α, and ECV304 showed both low baseline and stimulated ICAM expression. Taken together, the data show that hTERT(+) EC lines continuously passaged in vitro for 3–5 times the normal replicative life span of primary ECs exhibit both the functional and differentiated phenotype of early passage, primary ECs.

hTERT-ECs Maintain Growth and Cell Cycle Control Patterns Similar to Parental ECs—We investigated the growth of hTERT(+) EC lines and found no significant differences in their growth rates compared with parental ECs prior to senescence. The growth curves shown in Fig. 5A are representative of all large vessel hTERT-EC lines and indicate no alteration in growth rates with continuous passage in vitro. Although mitotic rates were not analyzed in detail, simultaneous growth curves of parental HDMECs at PDL 10 and hTERT(+) HDMECs at PDL 65 generated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (30) showed no significant differences during a 7-day period. Similar to previous studies in human skin fibroblast and RPE lines produced by hTERT expression (26, 27) hTERT(+) EC lines are contact-inhibited and exhibited normal pRb phosphorylation patterns in response to confluency-induced growth arrest (Fig. 5B). In addition, no changes in pRb phosphorylation patterns were observed upon growth arrest induced by either serum deprivation or hydroxyurea treatment when hTERT(+) ECs were compared with parental EC controls (Table II, data not shown). When anchorage-dependent growth was assessed using the soft agar assay, ECV304 cells formed colonies readily, whereas none of the hTERT-EC lines did (Fig. 5C). Taken together, these data suggest that, contrary to viral oncogene-
induced or spontaneously transformed cell lines, hTERT(+) EC lines do not exhibit the growth or cell cycle control patterns of neoplastic, transformed cells even after growing in vitro for 2–5 times their respective life spans.

G banding and cytogenetic analyses showed that both parental HDMEC preparations we used had a normal male, diploid karyotype, and this was maintained upon hTERT immortalization (Fig. 6). Tetraploidy was observed in one hTERT(+) HDMEC line, hTERT1, at PDL 140; it was not seen at PDL 50. Another hTERT(+) HDMEC line, hTERT3, had no tetraploidy at PDL 75. No aneuploidy was observed in any parental or hTERT(+) HDMEC line. On the other hand, karyotypic abnormalities were found in both large vessel parental ECs, as well as hTERT(+) large vessel ECs, and these abnormalities were similar to the polyploid changes observed in late passage fibroblasts and hTERT(+) fibroblast lines (26). Parental large vessel ECs had at least 40% aneuploidy at early PDL, which increased to nearly 100% aneuploidy by late PDL. All large vessel hTERT(+) EC lines also had 100% aneuploidy, and thus hTERT expression did not prevent or reverse the genomic instability observed in parental ECs before hTERT expression (see Table II).

Taken altogether, these results indicate that introduction of telomerase into normal human ECs in vitro does not lead to abnormal growth patterns, cell transformation, or genomic instability.

**Decreased Programmed Cell Death in hTERT(+) ECs**—Recent studies indicate that EC apoptosis is associated with important vascular remodeling patterns under both physiologic and pathologic conditions (45–50). Clarification of EC apoptotic mechanisms thus represents an important therapeutic strategy in the management of acute, chronic, and neoplastic diseases (51–53). Previous studies of human dermal fibroblasts showed that senescent cells are more resistant to apoptosis relative to young cells in vitro (54, 55). Therefore, we monitored the basal apoptotic rate in HDMECs and found that both early and late passage parental HDMECs showed lower nuclear fragmentation relative to mid passage HDMECs (Fig. 7A), with differences reaching statistical significance for PDL 15 versus both PDL 5 and 25. The effect of PDL on apoptosis in primary HDMEC cultures was verified by flow cytometric analysis of Apo2.7 positivity, reflecting the exposure of an early and specific mitochondrial apoptotic protein, 7A6 (38) (Fig. 7B). Two different hTERT-HDMEC lines showed statistically significant differences in baseline apoptotic rates versus each other, and both lines were comparable to early and late passage parental HDMECs (Fig. 7, A and B).
A transformed cell line did (left) as an example (Methods). None of our hTERT immortalization experiments (upper panel) showed comparable phosphorylation patterns to hTERT(+) ECs (lower panel). C, representative photomicrographs of colony formation in soft agar (see under “Materials and Methods”). None of our hTERT(+) EC strains (HAECs at PDL 127 used as an example) (right) formed colonies in soft agar, whereas the ECV304 transformed cell line did (left).

Next, we compared apoptosis after stimulation with several different EC apoptotic inducers using the two hTERT-HDMEC lines and late passage, presenescent parental HDMECs as controls. Four different conditions for inducing EC apoptosis all showed the same result: hTERT(+) HDMECs resisted apoptotic induction relative to primary HDMECs (Fig. 7, C and D). Except for TNF-α + actinomycin D induction in hTERT1 cells, both hTERT-HDMEC lines expressed statistically significant lower nuclear fragmentation versus controls in response to all treatments. Lipopolysaccharide + cycloheximide induction showed significantly decreased Apo2.7 expression in hTERT-HDMEC1 versus control, whereas other treatments did not reach statistical significance. The hTERT-HDMEC3 line that exhibited lower baseline apoptosis generally showed the lowest stimulated apoptotic rates. UV light-induced nuclear fragmentation and Apo2.7 expression appeared to reveal the most dramatic differences between primary and both hTERT HDMEC lines. Experiments assessing apoptosis resistance in large vessel hTERT(+) EC lines are under way, but preliminary studies using annexin V expression in HAECs after TNF-α + actinomycin D induction showed a similar apoptosis resistance (data not shown).

**DISCUSSION**

In using five different types of human ECs, we have demonstrated the general applicability of using ectopic expression of hTERT to bypass replicative senescence while maintaining EC phenotypic and morphogenetic characteristics in vitro. Upon stable transfection or retroviral transduction of hTERT, telomerase activity was detectable in all ECs, and telomere lengths decreased with time in culture and then stabilized. To date, both hTERT-expressing clones and mass cultures have achieved a PDL (PDL 50–160) 2.5–5 times that of parental or control vector-transduced cells (PDL 30–50) and therefore are considered “immortal” (43). All hTERT-EC clones and different lines have been continuously passaged without evidence of altered morphology or changes in growth patterns.

Detailed analysis of EC phenotypic patterns revealed several important characteristics about hTERT-ECs. First, the various parental and hTERT-EC lines are typical of “partially activated” endothelium in vivo (14); however, the cell adhesion molecule expression profiles of senescent primary ECs (Fig. 4 and Table II) resembled that of inflammatory tissue (7–9). Second, hTERT-ECs underwent morphogenetic differentiation to form capillary-like structures in response to extracellular matrix signals, whereas senescent and transformed ECs did not (Fig. 3), and this may reflect an age-related functional defect in angiogenesis in vivo (1, 2). Third, similar to previous reports in other cell types, hTERT expression per se did not induce EC cyrogenetic instability or a transformed phenotype (26, 27). Normal karyotypes were found in hTERT-HDMEC lines as in parental cells (Fig. 6 and Table II). hTERT-HUVEC clones and mass EC cultures were contact-inhibited, did not grow in soft agar, and exhibited appropriate pRb phosphorylation patterns in response to serum, cell density, and hydroxyurea-induced cell cycle arrest (Fig. 5, Table II). Finally, hTERT-HDMECs were more resistant to apoptotic induction than senescent parental controls or sham-transduced ECs (Fig. 7, C and D). Altogether, these results suggest that hTERT-ECs behave more like early passage, young ECs.

Telomere lengths in senescent ECs used in this study ranged between 5 and 7 kbp (data not shown), and our hTERT-HDMECs stabilized at 4–5 kbp (Fig. 1C). Previous studies in ECs (56), human fibroblasts (35), and various human cells (57) have shown that the mean TRF at senescence is approximately 4–7 kbp. hTERT immortalization of fibroblasts and RPE resulted in an increase and/or stabilization of telomere length to a size of >8 kbp (57). Thus, our hTERT-HUVEC clones with mean TRFs of 2–2.5 kbp (Fig. 1D) are well below those previously reported, and yet these cells bypassed senescence and continue to divide. Our results with hTERT(+) HUVEC clones are similar, however, to recent studies in hTERT(+) human fibroblast lines, which show a similar pattern of decreasing TRF with increasing PDL beyond the M2 crisis point (58). Possible reasons for these intrinsic differences in telomere lengths may relate to either cell type-specific mechanisms of telomere maintenance or threshold for functional telomerase of mass cell cultures versus individual clones.

Several studies have shown that genetic abnormalities occur in large vessel ECs (59, 60), but little is known about human microvascular EC cyogenetic instability. Our hTERT(+) HDMEC lines remained diploid after continuous passaging for 2–5 times their normal life spans. The tetraploid changes we observed in hTERT1 at PDL 140 are similar to many other human primary cells and immortalized cell lines that have been continuously grown in vitro for extended periods (76–78). However, the aneuploidy observed in both large vessel paren-
tal EC and hTERT-EC lines (Table II) indicates that hTERT expression does not appear to reverse these cytogenetic abnormalities. Because our hTERT-HDMEC lines showed a maintenance of normal karyotypes, these results suggest that hTERT expression per se does not induce chromosomal instability in human ECs. Recent studies in the mTR null mouse show critical interdependence of p53/p19, pRb/p16, p21, telomere dysfunction, and cell survival beyond the “genetic catastrophe” point (74, 75). Transformation into a neoplastic cell type in the mouse appears to depend on tolerance of genetic instability requiring certain adaptive responses, e.g. activation of oncogenes and/or telomerase, inactivation of tumor suppressors, etc. Our studies in hTERT(+) large vessel ECs show maintenance of normal growth rates and growth arrest patterns and absence of colony formation even in the face of cytogenetic instability at PDLs 2–4 times that of parental cells. These data suggest that a functional p53-dependent DNA damage checkpoint arrest pathway accompanying telomerase activation is important in preventing neoplastic transformation of large vessel human ECs.

The karyotypically normal hTERT(+) HDMECs exhibit a distinct survival advantage beyond that caused by hurdling the M1 senescence barrier (61), as these cells resist induction of apoptosis relative to senescent parental ECs under identical conditions. To our knowledge, this study is also the first to report EC apoptosis as a function of PDL, and our results indicate that senescent ECs were resistant to apoptosis relative only to midpassage primary ECs, not early passage cells, as reported previously in fibroblasts (54, 55). We used only confluent, growth-arrested cells for our apoptosis analyses as it is known that proliferating cells are more susceptible to apoptosis than are quiescent cells; however, unrepaird DNA and chromosomal damage are also known to trigger apoptotic induction (62–64, 74, 75). Only recently, it was found that DNA damage in the form of telomere shortening can be linked to either apoptosis or senescence, depending on a functional p53-dependent DNA checkpoint arrest pathway (65, 74, 75). A relationship between telomerase and apoptosis is apparent and it is known that proliferating cells are more susceptible to apoptosis than are quiescent cells; however, unrepaird DNA and chromosomal damage are also known to trigger apoptotic induction (62–64, 74, 75). Only recently, it was found that DNA damage in the form of telomere shortening can be linked to either apoptosis or senescence, depending on a functional p53-dependent DNA checkpoint arrest pathway (65, 74, 75). A relationship between telomerase and apoptosis is apparent and it is known that telomerase functions to repair chromosomal damage induced by a wide variety of different agents that cause

![Fig. 6. Karyotype of the hTERT(+) HDMEC line hTERT1 at PDL 50. Chromosomes were analyzed by GTW banding (see under “Materials and Methods”). The two parental HDMEC lines (at PDL 10 and 5, respectively) and both hTERT(+) HDMEC lines (hTERT1 and hTERT3 at PDL 50 and 75, respectively) all had normal diploid 46, XY male karyotype (20 metaphases for each). However, hTERT1 at PDL 140 were mostly tetraploid 92, XX YY (see Table II).](image)

![Fig. 7. HDMEC programmed cell death as measured by nuclear fragmentation cell death ELISA (A and C) and flow cytometric analysis of mitochondrial Apo2.7 positivity (B and D) (see under “Materials and Methods”). A and B, basal levels of apoptosis in primary parental HDMECs showed a dynamic change as a function of PDL with higher rates at mid passage (PDL 15, double asterisks) versus early (PDL 5) or late (PDL 25). Basal apoptotic rates of two different hTERT-HDMEC lines (hTERT1 and hTERT3) remained stable with increasing PDL, but the HDMEC3 line was significantly less apoptotic versus both primary and hTERT-HDMEC1 (single asterisk). C, following induction of apoptosis by treatment with either TNF-α (0.1 μg/ml) + actinomycin D (1 μg/ml), lipopolysaccharide (0.1 μg/ml) + cycloheximide (50 μg/ml), UVC exposure (255 nm for 0.3 min), or serum starvation (40 h), both hTERT-HDMEC lines exhibited significantly lower nuclear fragmentation (asterisks) versus senescent primary HDMEC controls. D, the same trend was found for Apo2.7 flow cytometry; however, only lipopolysaccharide-induced apoptosis in hTERT-HDMEC1 reached statistical significance (asterisk). Statistical significance, <0.05. Experiments were repeated three times on hTERT-HDMECs. Results shown are representative of three experiments.](image)
DNA strand breaks (66–69). Telomerase could thus be influencing nuclear fragmentation events observed in apoptosis, and several other studies support an inverse relationship between telomerase activity and programmed cell death (70–73).

Our results also support an inverse relationship between telomerase activity and apoptosis. The mechanism(s) of apoptotic resistance in telomerase-activated ECs is unclear, and further studies are in progress to clarify it. From the perspective of both potential therapeutic benefit and clarification of these possible mechanistic pathways, the development of an in vivo angiogenesis and vascular remodeling system incorporating hTERT(+) HDMECs becomes of paramount importance, and such studies are currently under way.

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