Functional and gene expression analysis of hTERT overexpressed endothelial cells

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Abstract: Telomerase dysfunction contributes to cellular senescence. Recent advances indicate the importance of senescence in maintaining vascular cell function in vitro. Human telomerase reverse transcriptase (hTERT) overexpression is thought to lead to resistance to apoptosis and oxidative stress. However, the mechanism in endothelial lineage cells is unclear. We tried to generate an immortal endothelial cell line from human umbilical vein endothelial cells using a no-virus system and examine the functional mechanisms of hTERT overexpressed endothelial cell senescence in vitro. High levels of hTERT genes and endothelial cell-specific markers were expressed during long-term culture. Also, angiogenic responses were observed in hTERT overexpressed endothelial cell. These cells showed a delay in senescence and appeared more resistant to stressed conditions. PI3K/Akt-related gene levels were enhanced in hTERT overexpressed endothelial cells. An up-regulated PI3K/Akt pathway caused by hTERT overexpression might contribute to anti-apoptosis and survival effects in endothelial lineage cells.

Keywords: endothelial, telomerase, senescence, oxidative stress, anti-apoptosis, PI3K/Akt pathway

In vitro, normal somatic cells undergo a non-dividing state termed cellular replicative senescence (Harley et al 1990; Hastie et al 1990; Wright and Shay 1992). The erosion of telomeres, a reverse transcriptase synthesize telomeric DNA, has been suggested to contribute to cellular replicative senescence (Greider 1990; Nakamura et al 1997). It is necessary to increase the number of human endothelial progenitor cells (EPCs) in vitro to obtain large numbers for research and cell transplantation. A potential obstacle to this increase, however, is the fact that EPCs, as well as other somatic cells, have a limited replication lifespan.

Human telomerase reverse transcriptase (hTERT)-transduced differentiated endothelial cell (ECs) exhibit neither evidence of malignant transformation nor loss of functional and morphologic characteristics of parental cells (Yang et al 1999). These findings support the stability of endothelial lineage cells after hTERT overexpression. We previously reported on the therapeutic potential of adenoviral hTERT overexpressed endothelial lineage cells in an ischemic hindlimb mouse model (Murasawa et al 2002). However, ectopic hTERT expression by adenovirus transduction was limited to 4 weeks and did not lead to immortal EPCs. We had expected that the enhanced regenerative activity of EPCs by hTERT transduction would provide a novel therapeutic strategy for potential neovascularization in patients with severe ischemic disease. However, the mechanism by which hTERT-transduced EC lines appear more resistant to programmed cell-death and exhibit a survival advantage beyond replicative senescence is elusive. Thus, further investigation of the possible mechanistic pathways is needed.

Here we perform gene transfer with a no-virus system to transduce hTERT genes into human umbilical vein endothelial cells (HUVECs), one of the endothelial lineage

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cell models, to investigate the relation between senescence and telomerase in vitro, determine the possible mechanistic pathways, and investigate the characteristics of these cells for future clinical application.

Materials and methods

Reagents

Recombinant human $VEGF_{165}$ and bFGF were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Hoechst 33342 solution was purchased from Dojin Chemical Laboratory (Kumamoto, Japan).

Cell culture and gene transfer

HUVECs were grown in endothelial cell growth media EGM-2 Bullet kit (CAMBREX, Walkersville, MD, USA) supplemented with 2% fetal bovine serum (CAMBREX). The cDNA encoding hTERT was cloned downstream of the human cytomegalovirus (CMV) promoter in pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) with Lipofectamine™ 2000 (Invitrogen). Forty-eight hours after transfection, the transfected-cell populations were selected with 600 μg/mL of G418 (GIBCO, Grand Island, NY). Cells transfected with empty vectors were used as controls. Population doublings (PD) were calculated as follows: PD = log (number of cells obtained/initial number of cells)/log 2.

RT-PCR for telomerase transcripts

Total RNA was isolated with an RNA extraction kit (Ambion, Austin, TX, USA). DNase digestion was performed after RNA extraction. cDNA was synthesized by AMV First-strand Synthesis System (Takara Bio, Shiga, Japan). PCR reaction of hTERT and endothelial markers were performed by a system according to the manufacture (BD Biosciences, San Diego, CA and Applied Biosystems, Foster City, CA, USA). The primers for RT-PCR are shown in Table 1.

Senescence-associated (SA)-Galactosidase (Gal) Staining

4×10^4 cells were plated on 12-well plates pre-coated with fibronectin and cultivated in growth medium for 24 hours. Senescence was investigated by a SA-Gal activity assay system according to the manufacture (CALBIOCHEM, San Diego, CA, USA). Quantification of SA-Gal–positive cells was obtained by counting five random fields per dish and assessing the percentage.

Proliferation assay

3.5×10^3 cells grown in 96-well plates pre-coated with fibronectin were cultivated in growth medium for 24 hours then in basal medium for another 24 hours. Then the culture medium was changed to basal medium in the presence of angiogenic cytokines for 24 hours. Proliferative activity was evaluated using the MTS assay (Promega, Madison, WI, USA).

Apoptosis assay

1×10^5 cells grown in 12-well plates pre-coated with fibronectin were cultivated in growth medium for 24 hours then in basal medium for another 24 hours. To detect the frequency of cellular apoptosis, fluorescence-labeled Annexin-V-FLUOS staining of transduced HUVECs was performed according to manufacture's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Quantification of Annexin-V positive cells was obtained by counting 5 random fields per dish and assessing the percentage.

Oxidative stress detection assay

1×10^5 cells were grown in 12-well plates pre-coated with fibronectin cultivated in growth medium. After 24 hours' incubation, the cells were incubated with 1mM H_2O_2 in HEPES buffer for 1 hour. Then the medium was changed to growth medium for another 3 hours. Detection of oxidative damage in live cells was performed using the Image-iT™ LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Molecular Probes, Eugene, OR, USA). Quantification of ROS detected cells was obtained by counting 5 random fields per dish and assessing the percentage.

Gene array analysis

Targeted cDNA arrays designed to analyze endothelial cell biology, angiogenesis, apoptosis, cell cycle, and PI3K/Akt pathway (GEArray HS-036, HS-009, HS-603, and HS-058)

Table 1 List of primer sequences for PCR

Molecule	Sense (5'–3')	Antiense (5'–3')
hTERT	CACCTCACCTCACCCACGCGAAA	CCAAAGAGTTTGCGACGCATGTT
hCD31	AGGACATCCATGTTCCGAGA	TGAACCGTGTCTTCAGGTTG
hKDR	CCCTGCCGTGTTGAAGAGTT	GGACAGGGGGAAGAACAAAA
heNOS	TTACCATGGCAACCAACGTC	AAAAGCTCTGGGTGCGTATG
hGAPDH	GCCCCAGCAAGAGCACAAGA	TAGGCCCCTCCCCTCTTCAA

were purchased from SuperArray Bioscience Corporation (Frederick, MD, USA). Hybridization was quantified using a Luminescent Image Analyzer LAS-3000 (Fujifilm, Tokyo, Japan) and data analysis was performed using Science Lab 2001 Image Gauge (Fujifilm) software. The background was subtracted from each representing dot signal, and values were normalized according to the GAPDH signal.

Statistical analysis

All values are expressed as mean ± SD. Comparisons of results between different groups were performed using Student's t test.

Results

hTERT transduced HUVECs expressed telomerase gene during long term culture

hTERT transduced HUVECs (Td/TERTs) were isolated from several donors cell sources. RT-PCR analysis showed that telomerase gene expression was maintained during long term culture, and the level achieved in Td/TERTs was comparable to that expressed by the 293 human embryonic kidney tumor cell lines (Figure 1A). By contrast, mock transduced HUVECs (Td/mocks) did not express endogenous telomerase at early cultures (data not shown). Further, the transduced HUVECs expressed endothelial markers such as CD31, KDR, and eNOS during long term culture (Figure 1B). Sequences of PCR primers are shown in Table 1.

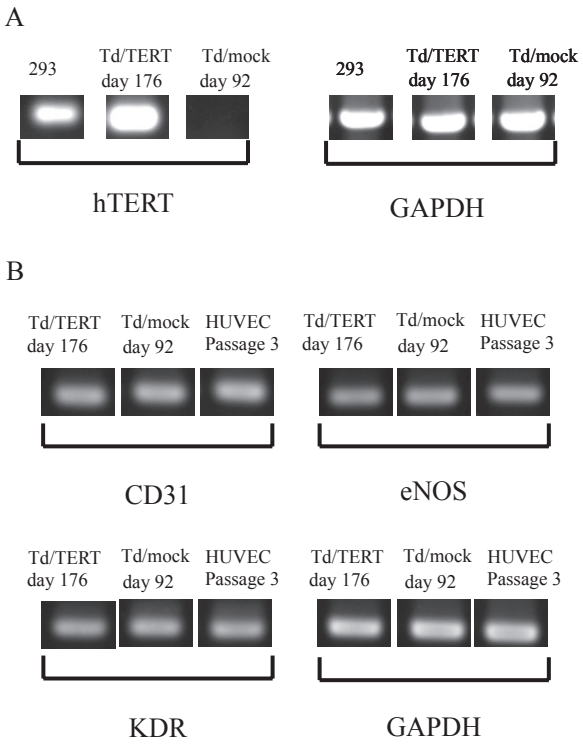

Figure 1 hTERT transduced HUVECs expressed telomerase genes during long-term culture. RNA samples from transduced HUVECs were analyzed by RT-PCR for gene expression. (**A**) Expression of hTERT gene in 293 cells served as the positive standard. (**B**) Expression of endothelial markers.

Transduced HUVEC lines maintained endothelial characteristics

Phenotypes of these cell lines displayed serum-dependence for growth (Figure 2A) and normal contact inhibition

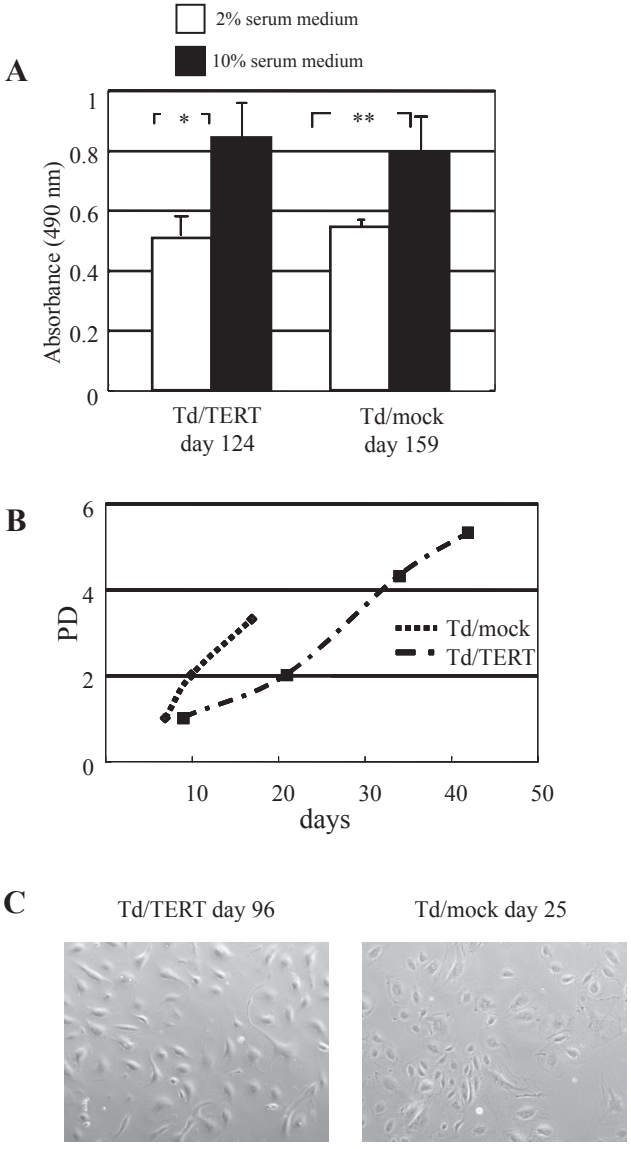

Figure 2 Transduced HUVEC lines maintained endothelial characteristics. (**A**) Growth of transduced HUVECs, Td/mock (day 159) and Td/TERT (day 124), was inhibited in low serum (*$p < 0.001$, **$p < 0.05$). (**B**) Growth curves of transduced HUVEC. About in a month, most transduced HUVECs showed almost a typical senescent endothelial morphology and stopped continuous growth. However, hTERT overexpression allowed the maintanance of a relatively normal endothelial morphology during the aging process. (**C**) Phase microscopy demonstrated endothelial morphology of Td/TERTs (day 96) and Td/mocks (day 25). Original magnification ×100.

(data not shown). Thus, the transduced HUVEC lines showed no signs of tumorigenic transformation. About in a month, transduced HUVECs ordinary entered the growth-arrested state (Figure 2B), and these phenotypes developed an enlarged and flattened morphology (Figure 2C, Td/mocks [day 25]). In contrast, the Td/TERTs (day 96) maintained a relatively normal endothelial morphology during the aging process (Figure 2C). However, some Td/mocks survived more than a month and maintained the same normal endothelial morphology as the Td/TERTs (data not shown).

Transduced HUVEC senescence evaluated by SA-Gal staining

The cytochemical senescent phenotype was also examined using SA-Gal activity. As expected, hTERT expression facilitated the delay in senescence (Figure 3A, B). During the aging process, transduced HUVECs revealed SA-Gal activity. However, the ratio to total cells in Td/mocks (day 32) was significantly increased compared with that in Td/TERTs (day 105) (41% ± 13% vs 7% ± 4%, $p \leq 0.001$). These results indicated that hTERT overexpression delayed the cells from entering their senescent state.

hTERT overexpression exceeded mitogenic potential.

Endothelial cell lines have a mitogenic response to angiogenic cytokines. To determine whether Td/TERTs produced angiogenic responses on stimulation by growth factors, we performed angiogenic proliferation assays by treating transduced HUVECs with different cytokines in $VEGF_{165}$ and bFGF while applying serum-depleted basal EBM-2 medium. As shown in Figure 3C, D, when treated with $VEGF_{165}$ (Figure 3C) and bFGF (Figure 3D), MTS assay showed significantly increased proliferation in Td/TERTs

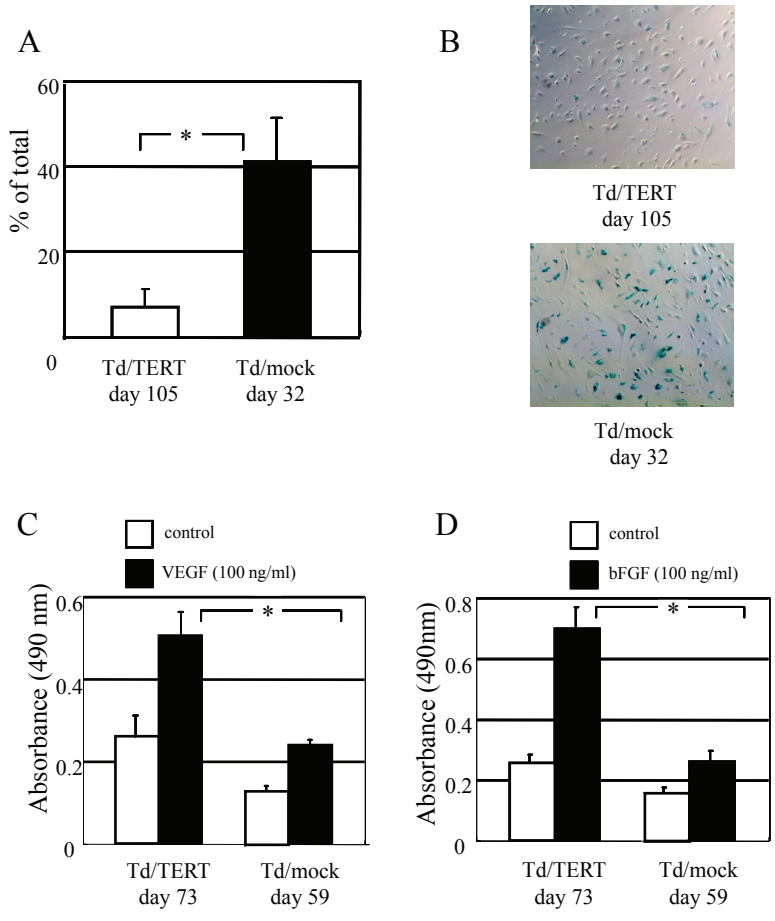

Figure 3 Senescence in transduced HUVECs was evaluated by SA-Gal staining. (**A**) Quantification of SA-Gal–positive cells in transduced HUVECs obtained by counting 5 random fields per dish ($p < 0.001$ vs Td/mocks). (**B**) Representative photomicrographs show SA-Gal–positive cells (blue) in Td/TERTs (day 105) and Td/mocks (day 32). Original magnification x100. hTERT overexpression exceeded mitogenic potential. (**C**) Proliferative activity assay response to $VEGF_{165}$. (**D**) Proliferative activity assay response to bFGF. The increase in mitogenic response to angiogenic cytokines of Td/TERTs (day 73) was statistically significant compared with Td/mocks (day 59) (VEGF: $p < 0.001$; bFGF: $p < 0.001$ vs Td/mocks).

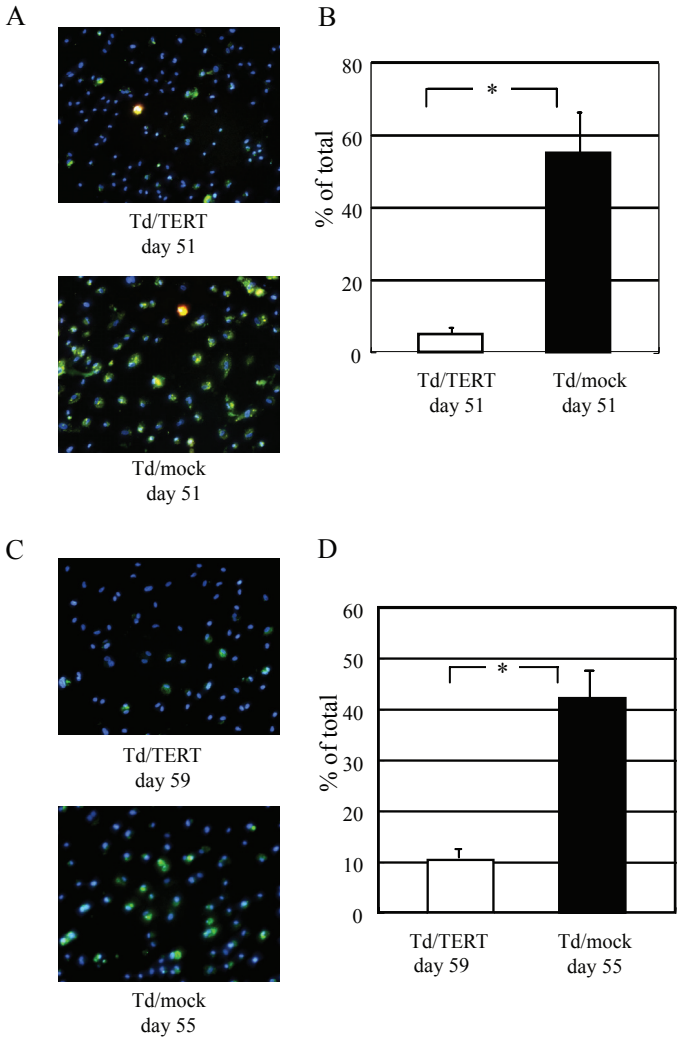

Figure 4 hTERT transduction contributes to cell survival. (**A**) Representative photomicrographs show Annexin-V (green), propidium iodide (red), and nuclei were stained with blue-fluorescent Hoechst 33342. Original magnification x100. (**B**) Quantification of apoptosis-induced. The number of apoptotic cells was significantly increased in Td/mocks ($p < 0.001$) vs Td/TERTs. (**C**) Oxidative stress measurement in live cells. Representative photomicrographs show the cells stained with carboxy-H_2DCFDA (green), corresponding to oxidized cells by ROS, and nuclei were stained with blue-fluorescent Hoechst 33342. Original magnification x100. (**D**) Quantification of oxidatively damaged cells. The oxidatively damaged cells significantly increased in Td/mocks ($p < 0.001$) vs Td/TERTs.

(at day 73) than that in Td/mocks (at day 59) (VEGF; 0.506 nm ± 0.061 nm vs 0.239 nm ± 0.013 nm, $p < 0.001$, bFGF; 0.697 nm ± 0.067 nm vs 0.261 nm ± 0.027 nm, $p < 0.001$). These data suggest that Td/TERTs generate more remarkable angiogenic responses to angiogenic cytokines, perhaps as part of their general endothelial function, than that of Td/mocks.

hTERT transduction contributes to cell survival

To confirm whether hTERT modulates cell survival, cell apoptosis and oxidative damage were evaluated in transduced HUVECs. Strong Annexin-V (green) staining, corresponding to apoptotic cells, was observed in Td/mocks (Figure 4A). The staining indicated that serum starvation-induced apoptosis was markedly reduced in Td/TERTs (Figure 4, B) (4.9% ± 1.5% vs 54.9% ± 11.0%, $p < 0.001$). Furthermore, strong carboxy-H_2DCFDA (green) staining, corresponding to oxidized by ROS, was observed in Td/mocks (Figure 4C). The oxidatively damaged cells significantly increased in Td/mocks (10.3% ± 2.2% vs 42.1% ± 6.7%, $p < 0.001$) (Figure 4, D). Staining in blue indicated nuclei by Hoechst 33342 (Figure 4A, C) Taken together, these results suggest that hTERT overexpression in HUVECs contributes to resisting apoptosis and oxidative damage.

Gene expression up-regulated in hTERT HUVEC

Finally we examined gene levels of transduced HUVECs. To explore the genes involved in hTERT-induced cell survival,

we applied cDNA microarray to transduced HUVECs. We compared the endothelial cell biology, angiogenesis, PI3K/Akt signaling pathway, apoptosis, and cell cycle gene expression profiles of Td/mocks (day 46) and Td/TERTs (day 60). Microarray analysis revealed that hTERT overexpression altered expression of various genes related to apoptosis, cell survival, and signal transduction molecules. As shown in Table 2, survival signals such as PI3K/Akt related gene levels were enhanced in hTERT overexpressed HUVECs.

Discussion

Functional changes in senescent endothelial cells in vivo may play an important role in the pathophysiology of age-associated vascular disorders. Thus, recovery of endothelial cell profiles could have possible clinical advantages in some cases. Recently, transduction of hTERT has been used to generate immortal cells. Various approaches have been used, none of which convert the cells to a transformed phenotype (Bodnar et al 1998; Jiang et al 1999; Morales et al 1999;

Table 2 Gene array analysis of transduced HUVECs

Gene bank	Symbol	Gene name	Description	
			AKT and PI3K Family Members and Their Regulators	
NM021158	C20orf97	TRB3	Chromosome 20 open reading frame 97	++
NM002074	GNB1	GNB1	Guanine nucleotide binding protein (G protein), beta polypeptide 1	++
NM000883	IMPDH1	IMPDH1	IMP (inosine monophosphate) dehydrogenase 1	+++
NM005541	INPP5D	SHIP	Inositol polyphosphate-5- phosphatase,145kDa	++
NM014221	MTCP1	MTCP1	Mature T-cell proliferation 1	++
NM006218	PIK3CA	p110alpha	phosphoinositide-3kinase, catalytic, alpha polypeptide	++
NM006219	PIK3CB	p110beta	phosphoinositide-3kinase, catalytic, beta polypeptide	++
NM003629	PIK3R3	p55 gamma	Phosphoinositide-3-kinase,regulatory subunit, polypeptide 3 (p55, gamma)	+++
NM181897	PPP2R3A	PPP2R3A	Protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha	++
NM006246	PPP2R5E	PPP2R5E	Protein phosphatase 2, regulatory subunit B, (B56), epsilon isoform	++
NM002737	PRKCA	PKC alpha	Protein kinase C, alpha	+++
			PI3K-Dependent/AKT-Dependent Pathways	
NM002093	GSK3B	GSK3	Glycogen synthase kinase 3 beta	++
NM005923	MAP3K5	MEKK5/ASK1	Mitogen-activated protein kinase kinase kinase 5	+++
NM005938	MLLT7	AFX1	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophia); translocated to,7	++
NM002467	MYC	c-Myc	V-myc myelocytomatosis viral oncogene homolog (avian)	+++
NM003998	NFKB1	KBF1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	+++
NM020529	NFKBIA	IKBA/MAD-3	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	+++
NM001278	CHUK	IKKA/IKK1	Conserved helix-loop-helix ubiquitous kinase	+++
NM002576	PAK1	Pak1	P21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	+++
NM012296	GAB2	GAB2	GRB2-associated binding protein 2	+++
			Apoptosis Panel for Monitoring Cell Programmed Death and Survival	
NM004536	BIRC1	NAIP	Baculoviral IAP repeat-containing protein 1 (Neuronal apoptosis inhibitory protein)	++
NM003810	TNFSF10	TRAIL	Tumor necrosis factor (ligand) superfamily member, 10	+
NM003701	TNFSF11	TRANCE	Tumor necrosis factor (ligand) superfamily member, 11	++
NM003811	TNFSF9	4-1BB-L	Tumor necrosis factor (ligand) superfamily member, 9	+
NM001561	TNFRSF9	4-1BB	Tumor necrosis factor receptor superfamily member, 9	+++
			Stress & Toxicity Panel for Monitoring the Type of Stress Induced	
NM002983	CCL3	MIP-1a/SCYA3	Chemokine (C-C motif) ligane 3 (Macrophage inflammatory protein 1-alpha)	+
NM021027	UGT1A9	UGT1A9	UDP glycosyltransferase 1 family, polypeptide A9	++

cDNA microarrays were performed to analyze the gene expression of transduced HUVECs. PI3K/Akt related gene levels were enhanced in Td/hTERTs compared with Td/mocks.

+: 2- to < 4-fold up-regulation in Td/hTERT, ++: 4- to < 10-fold up-regulation in Td/hTERT, +++: 10- or > 10-fold up-regulation in Td/hTERT compared with Td/mock.

Gagnon et al 2002; Yang et al 1999). Virus systems are the most efficient way to deliver genes to cells. However, the clinical applications of viral vectors are limited because of the intrinsic properties of viruses. Therefore, it seems that no-virus approaches of somatic gene delivery are preferable. In this regard, we tried to generate endothelial cell lines by ectopic expression of hTERT in HUVEC using a no-virus system.

Several interesting functional properties were noted in our experiments. First, Td/hTERT retained high level hTERT gene expression during long-term culture, and the cells stably expressed typical endothelial cell markers, including CD31, KDR, and eNOS. In agreement with other reports (Yang et al 1999; Nisato et al 2004; Freedman and Folkman 2004; Shao and Guo 2004), hTERT expression exhibited contact inhibition and serum-dependent cell proliferation, and did not show signs of tumorigenic transformation. Second, Td/hTERT cell lines had significant mitogenic activity in response to angiogenic cytokines compared with Td/mocks. Third, hTERT induced a delay in senescence and hTERT overexpressed cells appeared more resistant to stressed conditions. Fourth, a variety of genes related to cell survival were enhanced in Td/hTERT. In contrast to the immortalization shown in previous reports (Yang et al 1999; Nisato et al 2004; Freedman and Folkman 2004; Shao and Guo 2004), our system did not establish immortalized endothelial cells, but led to improved functional integrity as well as adenovirus transduction. For immortalization, Kiyono et al (1998) demonstrated that telomerase activity was not sufficient for immortalization of human kerationocyte or mammary epithelial cells. It likely that additional activities of antisenescent genes may be required to maintain functional integrity. Moreover, for gene transfer with potential therapeutic efficiency, immortalization is not necessarily valuable for clinical approaches.

Our results imply that telomerase inhibited functional alterations associated with senescence in endothelial cells. Thus we tried to study the molecular profile and mechanisms of apoptotic resistance and the ability to delay senescence.

Several factors, such as oxidative stress and DNA damage, have been shown to cause endothelial dysfunction. Recent studies showed that introduction of TERT into human vascular cells increased longevity and functional activity of endothelial cells (Yang et al 1999), suggesting that telomere function is necessary for endothelial function. Endothelial cell aging is linked to an increase in intracellular ROS formation, which in turn affects TERT localization and activity (Haendler et al 2004), and oxidative stress-caused perturbation of antioxidant mechanisms results in decreased telomeric integrity in endothelial cells (Kurz et al 2004). Moreover, reduction of nuclear TERT during endothelial cell aging increases the sensitivity of aged endothelial cells toward apoptotic stimuli (Assmus et al 2003). Thus recent studies have gradually elucidated the function of TERT in regulating a delay in endothelial cell senescence.

Our gene array data suggest that the PI3K/Akt-related gene levels were enhanced in Td/hTERT. The serine/threonine kinase Akt, activated by various growth and survival factors through a pathway that requires PI3K-dependent, has been shown to regulate multiple biological processes and also acts as an anti-apoptosis protein (Franke et al 1997; Crowder et al 1998; Eves et al 1998; Ulrich et al 1998). In case of endothelial cell, it has been shown to act downstream of angiogenic growth factors VEGF and angiopoietin, and contributes to endothelial cell survival, proper vessel development (Gerber et al 1998; Kontos et al 1998; Carmeliet et al 1999; Kim et al 2000; Papapetropoulos et al 2000), formation of vascular structures in vitro (Kureishi et al 2000), and regulates endothelial cell nitric oxide production in response to VEGF (Luo et al 2000). Recently, Zaccagnini et al (2005) demonstrated that telomerase mediates VEGF-dependent responsiveness in the rat ischemic hindlimb model. They introduced the idea that hTERT behaves as an angiogenic factor and a downstream effector of VEGF signaling, and that telomerase activity appears to be required for VEGF-dependent remodeling of ischemic tissue at the capillary and arteriole level. They hypothesized that the function of hTERT, at least in vascular cells, might be controlled by a molecular network of autoregulatory loops in which growth factor production plays an important role.

Furthermore, recent studies suggest that ROS works as a regulator of signal transduction (Griendling and Ushio-Fukai 2000). Nishida et al (2000, 2002) reported that heterotrimeric Gi/o proteins are putative target molecules of ROS and the βγ-subunit of G protein (Gβγ) liberated from Gi and Go by oxidative stress activates PI3K, which in turn leads to activation of Akt and ERK. They hypothesized that the intracellular signal transduction induced by the Gβγ of Gi and Go may protect cells against oxidative stress as part of their normal cellular function. It is possible that Td/hTERT obtained during normal cellular function against stress conditions, as well as stable nuclear TERT obtained during cell aging, might cause the activation of PI3K/Akt-related gene levels and protect cells against various stress conditions.

We are trying to determine the various potentials of hTERT in endothelial cells. The knowledge gained from the further study of telomerase overexpressed endothelial

cells might help explain effects on cell survival. Further mechanistic investigations could contribute to improvements in vascular disorders in terms of the selection of cell sources when patients undergo cell transplantation.

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Disclosures

None of the authors have any conflicts of interest to disclose.

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