Constitutive Human Telomerase Reverse Transcriptase Expression Enhances Regenerative Properties of Endothelial Progenitor Cells

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Background—The regulatory molecule for cell life span, telomerase, was modified by human telomerase reverse transcriptase (hTERT) gene transfer to investigate its effect on regenerative properties of endothelial progenitor cells (EPCs) in neovascularization.

Methods and Results—Telomerase activity was enhanced in hTERT-transduced EPCs (Td-TERTs) (1.2-fold versus no transduced EPCs [no-Td] and 1.2-fold versus GFP-transduced EPCs [Td/GFPs] at day 8; 5.2-fold versus no-Td and 4.8-fold versus Td/GFP at day 21, respectively). Mitogenic capacity in Td/TERTs exceeded that in Td/GFPs at day 8 (0.62±0.02 versus 0.53±0.01, respectively; P<0.01). Vascular endothelial growth factor–induced cell migration in EPCs was markedly enhanced by hTERT overexpression (Td/TERTs versus Td/GFPs, 292±12 versus 174±6 cells, respectively; P<0.01). hTERT overexpression has rescued EPCs from starvation-induced cell apoptosis, an outcome that was further enhanced in response to vascular endothelial growth factor. The colony appearance of totally differentiated endothelial cells (tdECs) was detected before day 30 only in Td/TERT, whereas no tdEC colonies could be detected in both Td/GFPs and no-Tds. Finally, we investigated in vivo transplantation of heterologous EPCs. Td/TERTs dramatically improved postnatal neovascularization in terms of limb salvage by 4-fold in comparison with that of Td/GFPs; limb perfusion was measured by laser Doppler (0.77±0.10 versus 0.47±0.06; P=0.02), and capillary density (224±78 versus 90±40 capillaries/mm²; P<0.01).

Conclusions—These findings provide the novel evidence that telomerase activity contributes to EPC angiogenic properties; mitogenic activity, migratory activity, and cell survival. This enhanced regenerative activity of EPCs by hTERT transfer will provide novel therapeutic strategy for postnatal neovascularization in severe ischemic disease patients. (Circulation. 2002;106:1133-1139.)

Key Words: telomerase ■ gene therapy ■ stem cells, endothelial ■ angiogenesis ■ ischemia

The plasticity of stem and progenitor cells is attracting the attention to regenerative application to many inherited and acquired diseases. The regenerative potential of bone marrow–derived endothelial progenitor cells (EPCs)1–3 has been previously demonstrated in animal models of myocardial ischemia4 and limb ischemia, via ex vivo expansion and incorporation into foci of neovascularization. Physiological evidence of neovascular function in these preclinical animal models disclosed an improvement in myocardial function or a high rate of limb salvage. Despite promising applications for tissue regeneration, the limited endogenous pool, the possible functional impairment associated with a variety of physiological and pathological phenotypes on clinical patients, and the finite replicative feature of EPCs for ex vivo expansion constitute potentially important liabilities for autologous transplantation. We hypothesized that gene transfer can be used to achieve phenotype modulation of EPCs to overcome this issue. Very recently, our laboratory has determined the impact of vascular endothelial growth factor (VEGF) gene transfer on certain properties of EPCs in vitro and the consequences of VEGF EPC transfer on neovascularization in vivo.5

Most somatic cells of humans and other mammals undergo a finite number of cell divisions, ultimately entering a nondividing state termed senescence.6–8 Loss of telomerase activity has been suggested to constitute the molecular clock that triggers cellular senescence.9,10 In contrast to somatic cells, true stem cells and germline cells highly express the
catalytic subunit of telomerase (human telomerase reverse transcriptase [hTERT]), thus maintaining telomerase activity and full replication of telomeric DNA; these cells (by definition) are thereby able to divide indefinitely. Although demonstrating regenerative potentials for vascular development, EPCs are not pluripotent, self-renewing stem cells, but rather lineage-committed progenitors, and are thus subject to a Hayflick life span via replicative senescence. Accordingly, we have deduced that constitutive expression of hTERT might induce delay in senescence and recover/ enhance regenerative properties of EPCs. Ectopic expression of the hTERT gene has been investigated as a means to bypass senescence; indeed, this strategy has been successfully to impart replicative immortality to fibroblasts and retinal pigment epithelial cells without converting either to a transformed neoplastic phenotype. The immortalized blood vessel–derived endothelial cells (ECs) similarly exhibited neither evidence of malignant transformation nor loss of functional and morphogenetic characteristics of the parental cells. Such hTERT-transduced (Td-hTERT) EC lines appeared more resistant to programmed cell death, exhibited a survival advantage beyond replicative senescence, and had improved NO production compared with that of control senescent cells.

These findings have encouraged consideration of potential therapeutic applications of hTERT gene transfer to achieve functional improvement in EPCs through delay in senescence and recovering/enhancing regenerative properties of EPCs.

Methods

EPC Culture and Gene Transfer

Total peripheral blood mononuclear cells were isolated from human volunteers by density-gradient centrifugation. After 4 days in culture, nonadherent cells were removed by washing with PBS, new media was applied, and the culture was maintained through day 7 or later. In the culture of EPCs after day 7, reseeding was performed once a week. Both Ad/hTERT (Ad5CMVe/β-actin pro/hTERT ΔE3) and Ad/GFP (Ad5CMV/GFP ΔE3) were provided by Geron Inc (Menlo Park, Calif). Briefly, the recombinant adenoviruses were constructed by homologous recombination between the parental virus genome and the expression cosmid cassette or shuttle vector as described. Established colonies were evaluated as tdECs by use of assays for 1,1′-diododecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (acLDL-DiI) incorporation and Matrigel tube formation. Incorporation of acLDL-DiI typically appears less intense in tdECs (stain moderately within 1 hour with 20 μg/mL acLDL-DiI) than in the less differentiated EPCs (stain strongly within 30 minutes with 5 μg/mL acLDL-DiI). Non-EC—including fibroblasts, myoblasts, and epiblasts—do not incorporate acLDL. Within 12 hours, tdECs form complete capillary-like tubes in Matrigel (Becton Dickinson Labware).

Murine Hindlimb Ischemia Model

The impact of EPC administration on therapeutic neovascularization was investigated in a murine model of hindlimb ischemia, by use of athymic nude or severe combined immunodeficient mice. Athymic nude mice (Jackson Labs, Bar Harbor, Maine), age 8 to 10 wks and weighing 17 to 22 g, were anesthetized with 160 mg/kg pentobarbital intraperitoneally for operative resection of one femoral artery, and subsequently for laser Doppler perfusion imaging (LDPI; Liscia). Immediately before euthanasia, mice were injected with an overdose of pentobarbital. One day after operatively induced hindlimb ischemia, the athymic nude mice, in which angiogenesis is characterizedly impaired, received an intravenous injection of 1.5 × 10⁴ culture-expanded EPCs transduced with Ad/hTERT (Td/hTERT) or Ad/GFP (Td/GFP). Tissue sections were stained for alkaline phosphatase by use of indoxyltetrazolium to detect capillary ECs, as previously described and were then counterstained with cosin.

Results

Time Course of hTERT Overexpression After Adenoviral Transduction

RT-PCR was performed to evaluate the hTERT expression level after adenoviral transduction. Endogenous hTERT was observed only in day-10 nontransduced EPCs (no-Td). On the other hand ectopic hTERT was highly expressed at day 10, 3 days after Ad/hTERT transduction, and was gradually reduced during cultivating EPCs. Reseeding was performed days 4 and 7 after isolation and once a week after day 7. (Figure 1).
Telomerase Activity After hTERT Overexpression

EPC gene modification was performed using adenovirus-encoding hTERT or green fluorescent protein (GFP; Ad/hTERT and Ad/GFP, respectively). Cultured EPCs, transduced on day 7 with Ad/hTERT and Ad/GFP (Td/hTERT or Td/GFP, respectively), as well as no-Tds, were assayed for telomerase activity at 1, 7, and 14 days after transduction (days 8, 14, and 21 in culture, respectively). Telomerase activity appeared robust in no-Td EPCs at day 8 but was dramatically reduced by days 14 and 21 (Figure 2A). After gene transfer, telomerase activity was enhanced in Td/hTERT EPCs (1.2-fold versus no-Td and 1.2-fold versus Td/GFP at day 8; 1.5-fold versus no-Td and 1.2-fold versus Td/GFP at day 14; 5.2-fold versus no-Td and 4.8-fold versus Td/GFP at day 21) (Figure 2B).

SA-β-Gal Activity in EPCs

The impact of hTERT expression on EPC senescence was evaluated by SA-β-Gal activity assay to confirm the result of telomerase activity. At day 8, SA-β-Gal-positive cells were equally rare among no-Td, Td/hTERT, and Td/GFP EPCs (0.13% ± 0.02 versus 0.07% ± 0.01 versus 0.27% ± 0.02; P = NS). At day 14, the proportion of SA-β-Gal positive cells was increased (∼5%) in no-Td and Td/GFP EPCs; among Td/hTERT, however, a very low proportion of senescent cells (no-Td versus Td/hTERT versus Td/GFP, 5.13% ± 0.30 versus 0.20% ± 0.02 versus 5.0% ± 0.30; P < 0.01) were observed. By day 21, the proportion of SA-β-Gal-positive cells in no-Td and Td/GFP EPCs was markedly increased versus Td/hTERT (no-Td versus Td/hTERT versus Td/GFP, 25.2% ± 0.4 versus 5.4% ± 0.2 versus 24.2% ± 0.5; P < 0.01).
The results suggest that hTERT expression facilitates delay in senescence.

**Effect of hTERT Overexpression on EPC Differentiation**

The finding of enhanced EPC differentiation also supports the contribution of hTERT overexpression. During long-term follow-up, colonies of tdEC were observed at day 30 only in Ad/TERT-transduced EPCs, whereas no-Td and Td/GFP cells detached before day 30, yielding no colonies in either group at this time point (Figure 4A). Incorporation of acLDL-Dil was evaluated to distinguish functional difference between tdECs and undifferentiated cells. The centrally located tdECs were identified by typically less intense uptake of acLDL-Dil versus the peripherally located, intensely stained, undifferentiated cells (Figure 4B). Colonies of tdECs formed sheetlike monolayers; the maximum number of colonies averaged 38 per 35-mm well (Figure 4C). The tdECs also formed capillary-like structures when reseeded in Matrigel (Figure 4D). The tdECs were equivalent to control differentiated ECs (human umbilical vein ECs and human microvascular ECs), both in terms of capillary-like response in Matrigel and of FACS analysis of endothelial surface molecule expression (data not shown). Follow-up (40 days) of Td/hTERT and Td/GFP EPCs disclosed no evidence of neoplastic transformation, including neither loss of contact inhibition nor unchecked cellular proliferation.

**EPC Mitogenic and Migratory Activity After hTERT Overexpression**

The impact of hTERT overexpression on regenerative potential was apparent from analysis of angiogenic profiles in hTERT overexpressing EPCs. MTS assay demonstrated that mitogenic potential after Td/hTERT transduction exceeded that in Td/GFP at day 8 (0.62 ± 0.02, 0.53 ± 0.01, respectively; \( P < 0.001 \)) (Figure 5A). Similarly, migratory activity in EPCs after hTERT transduction was analyzed in a modified Boyden chamber assay. VEGF-induced cell migration was markedly enhanced by hTERT overexpression (Td/hTERT versus Td/GFP, 292 ± 12 versus 174 ± 6 cells/4 fields, respectively; \( P < 0.001 \)) (Figure 5B).
EPC Survival After hTERT Transduction

The effect of hTERT modulation on EPC resistance to apoptosis was also evaluated. Immunofluorescent staining with Annexin-V and Hoechst33342 established that starvation-induced EPC apoptosis was markedly reduced after hTERT gene transfer. MTS assay (shown in Figure 5A) also supported these results. This outcome was amplified after VEGF administration (Figure 6).

Physiological Impact In Vivo After Transplantation of hTERT-Transduced EPCs

Given the impact on regenerative features, EPC transplantation was performed to assess the corresponding physiological impact in vivo after hTERT gene modification. After 1-week ex vivo expansion, 1.5×10⁴ human EPCs were transduced with Ad/TERT or Ad/GFP and administered intravenously to athymic nude mice with unilateral hindlimb ischemia (n=18 each). Compared with mice transplanted with Td/GFP, mice transplanted with Td/TERT demonstrated enhanced perfusion measured by LDPI (0.77±0.10 versus 0.47±0.06 in arbitrary units measured by LDPI; P=0.02) (Figure 7B). The physiological relevance of this finding was underscored by the fact that salvage of the ischemic limb was significantly improved among mice transplanted with Td/TERT versus Td/GFP (P<0.01) (Figure 7A). Capillary density, evaluated in histologic sections retrieved at day 28 from ischemic hindlimb muscle, was markedly increased in mice receiving Td/TERT versus Td/GFP (224±78 versus 90±40 capillaries/mm²; P<0.01) (Figure 7D). Animals treated with Td/TERT or Td/GFP EPCs disclosed no evidence of neoplastic transformation.

Discussion

We have challenged the otherwise inevitable reduction of telomerase activity in human EPCs by performing gene transfer to achieve constitutive expression of hTERT in these somatic progenitor cells. Constitutive expression of hTERT...
led to conservation of telomerase activity and delay in senescence, as well as enhanced EPC regenerative properties, including mitogenesis, migration, and EPC survival. EPC differentiation colony assay also disclosed distinct consequences of hTERT transduction of EPCs. Differentiated colonies appeared only among hTERT-expressing EPCs. One possible mechanism of the hTERT effect on EPC differentiation may involve delay in senescence related to cell culture shock. Aging is one of the factors that might affect senescence; eg, EPCs from older people might go into senescence more rapidly than do EPCs used in the current experiments. Further investigation concerning time course in EPC senescence should be necessary. The in vivo experiments described here may be interpreted to constitute proof of concept that indirect gene transfer may facilitate therapeutic applications of EPC transplantation. The dose of EPCs used in the current in vivo experiments was 30 × less than that required in previous experiments designed to improve the rate of limb salvage. Moreover, GFP overexpression did not affect on EPC functions because of the equivalent finding of GFP-transduced EPCs and nontransduced EPCs with cell senescence and migratory and mitogenic activities. Thus, Td/hTERT EPC gene transfer constitutes one option to address the relative paucity of EPCs that can be isolated from peripheral blood before ex vivo expansion and subsequent autologous readministration.

We have questioned if hTERT-expressing cells acquired characteristics of cancer cells, such as chromosomal abnormalities, anchorage-independent growth in culture, or tumorigenicity in immunodeficient mice. There was no observation of such neoplasticity, however, after hTERT transduction by adenovirus constructs. The ectopic hTERT expression by adenovirus transduction was limited within 4 weeks and did not bring immortalization on EPCs. The stability of endothelial lineage cell after hTERT overexpression was also supported by the findings that hTERT-transduced differentiated ECs exhibited neither evidence of malignant transformation nor loss of functional and morphogenetic characteristics of the parental cells. We are paying attention to every possibility for applying this approach to any experimental or clinical application.

The augmented neovascularization observed after therapeutic transplantation of hTERT overexpressing EPCs is consistent with the favorable impact of hTERT gene transfer on the angiogenic profiles of EPCs in the in vitro experiments. As foci of injured organs are exposed to diverse physiological and pathological stress, replication and differentiation of certain cells (particularly stem and/or progenitor cells) may be required to initiate and complete tissue regeneration. The concept of “rejuvenating” EPCs via delay in senescence and enhanced regenerative properties may thus have therapeutic implications for vascular disorders, including myocardial ischemia and lower extremity, in which the viability of these and fully differentiated ECs is recurrently subjected to a variety of individual and environmental stress factors.

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