Decreased B16F10 Melanoma Growth and Impaired Vascularization in Telomerase-deficient Mice with Critically Short Telomeres

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ABSTRACT

Endothelial cell function and angiogenesis are modulated by aging. However, the underlying molecular mechanisms are largely unknown. Here we show that in telomerase-deficient mice Terc−/−, short telomeres result in a sharp decrease in angiogenesis in both Matrigel implants and murine melanoma grafts. In the latter model, decreased microvessel counts in late generation Terc−/− mice led to diminished tumor cell proliferation and increased tumor cell apoptosis, resulting in a lower tumor growth rate. Our results indicate that telomere length is a key molecular determinant of angiogenic potential in vivo and that telomere length modifiers and telomerase inhibitors could be useful antiangiogenic agents.

INTRODUCTION

Angiogenesis, the sprouting of new blood vessels from preexisting ones, is required for embryogenesis, adult female reproductive cycles, and wound healing (1, 2). The angiogenic process is tightly regulated by proangiogenic and antiangiogenic factors, and by interactions between endothelial cells and their microenvironment (3). Previous evidence suggests that endothelial cell function and angiogenesis are modulated with aging, as also described for the immune system and other tissues (4). Long-term culture of primary endothelial cells results in morphological and functional changes that resemble those described for senescent fibroblasts (5–9). Moreover, delayed wound healing, delayed ischemic tissue revascularization, and decreased tumor-induced angiogenesis have all been reported in aged mice (10–13). However, little is known about the molecular basis of the aging process. Multiple interrelated factors, such as cumulative oxidative stress, faulty DNA repair, and telomere shortening, may be responsible for age-associated cellular dysfunction (14). In this regard, a direct correlation between telomere length and replicative potential has been observed in human fibroblasts (15, 16). Furthermore, ectopic expression of telomerase reverse transcriptase, the cellular enzyme that synthesizes telomeres de novo using an associated RNA molecule as a template (Terc; Ref. 17), prevents the senescent phenotype by maintaining telomere length (18). Conversely, absence of telomerase activity in Terc−/− mice results in telomere shortening, increased end-to-end fusions, and leads to premature replicative exhaustion of highly proliferative tissues (19–23).

MATERIALS AND METHODS

Mice. Terc−/− mice in two genetic backgrounds were described elsewhere (19–23). Terc−/− mice in the C57BL6 background (BL6) become infertile after four generations, whereas mixed background (60% C57BL6, 37.5% 129Sv, and 2.5% SJL) Terc−/− mice (mixed background) can be crossed for up to six generations before infertility is observed. For each experiment, five to six 2–3-month-old sex-matched mice of each genotype were used.

Isolation of Bone Marrow Cells and Splenocytes. Bone marrow single-cell suspensions were obtained from bone marrow spiculae by flushing femurs with PBS. To obtain splenocytes, gentle pressure was applied onto spleens sandwiched between nylon screens using a syringe plunge. Erythrocytes were lysed by osmotic shock. Cell viability by trypan blue exclusion was >95% in all of the experiments.

In Vitro Proliferation of Splenocytes ([3H]Thymidine Incorporation). Splenocytes (2 × 10⁶) in 200 μl of 10% FCS RPMI supplemented with mitogens (10 μg/ml LPS or 5 μg/ml con A) were incubated with [3H]thymidine for 18 h starting at 24, 48, and 72 h after addition of mitogens. Cells were then harvested onto glass fiber filter strips (Wallac, Turku, Finland) using an LKB Wallac 1295–001 Cell Harvester and proliferation measured as cpm using an LKB 1205 Rackbeta Liquid Scintillation Counter (ICN, Costa Mesa, CA). All of the results are averages of quadruplicates. Parallel control non-stimulated lymphocytes were included in all plates.

Flow-Fluorescence in Situ Hybridization. Telomere length of freshly isolated splenocytes and bone marrow cells was determined by quantitative fluorescence in situ hybridization using a FITC-conjugated telomeric PNA probe as described (27). Briefly, cells were resuspended in hybridization buffer [formamide 70%, 20 mM Tris (pH 7.2), and 1% BSA] denatured at 80°C for 10 min, and incubated with a telomere-specific PNA probe for 2 h at room temperature. After centrifugation, cells were washed in 70% formamide, 10 mM Tris (pH 7.2), 0.1% BSA, and 0.1% Tween 20, incubated in RNase and propidium iodide for 2 h, and fluorescence of G1-G0 nuclei measured in a Coulter EPICS XL flow cytometer using SYSTEM 2 software. Background fluorescence was calculated for each sample in parallel using a probe-free hybridization solution and subtracted from the probe value.

TRF Analysis. Agarose plugs containing 5 × 10⁶ freshly isolated splenocytes or bone marrow cells were incubated with proteinase K overnight, digested with MboI, and resolved in a pulse-field electrophoresis chamber for 552

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3 The abbreviations used are: Terc, telomerase RNA component; TRF, telomere restriction fragment; BFGF, basic fibroblast growth factor; LPS, lipopolysaccharide; DAPI, 4′,6-diamido-2-phenylindole; BrdUrd, bromodeoxyuridine; con A, concanavalin A; TUNEL, terminal deoxyuridine transferase (TdT)-mediated nick end labeling.
23 h. DNA was then transferred to Hybond membranes and hybridized with a \(^{32}\)P-labeled telomere-specific probe, as described (19).

**Matrigel Assay.** The general procedure used was described by Passaniti et al. (28), including indirect quantitation with minor modifications. Briefly, 500 µl of Matrigel (Becton Dickinson, San Jose, CA) containing 100 ng/ml bFGF from Pharmacia (Nerviano, Italy) and 64 units/ml heparin (Sigma Chemical Co., St. Louis, MO) were injected into the abdominal s.c. tissue of mice along the peritoneal midline. Mice were killed 7 days after injection, Matrigel pieces excised, weighed, and homogenized in Drabkin’s Reagent (Sigma Chemical Co.) with a glass douncer, centrifuged (1000 \(x\) g; 10 min), and supernatants filtered through 45-µm Millipore filters (Millipore, Molsheim, France). Hemoglobin content was measured at 540 nm.

**Tumorigenesis Assay.** B16F10 murine melanoma cells were obtained from Pharmacia and cultured in RPMI 1640 with 10% FCS (both from BioWhittaker, Walkersville, MD), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. Mice were s.c. inoculated in both flanks with 10^5 B16F10 cells each in 150 µl sterile PBS. Tumor diameters were measured every other day with a slide caliper and tumor volume calculated using the formula: volume (mm\(^3\)) = width\(^2\) (mm\(^2\)) \times length (mm) \times 0.52. Three weeks after inoculation, mice were killed by CO\(_2\) suffocation, tumors excised, and snap-frozen in Tissue Freezing Medium (Jung, Nussloch, Germany).

**Quantification of Tumor Microvessels by Immunofluorescence.** For microvessel localization, 6-µm cryostat sections were fixed in cold acetone for 2 min, blocked with 2% BSA/10% goat serum in PBS for 60 min at 37°C, and incubated with a monoclonal anti-CD31 rat antibody (BD Biosciences, Heidelberg, Germany) for 1 h at room temperature. After three washes in PBS, slides were additionally incubated with Cy3-conjugated mouse antirat IgG (Jackson Immunoresearch, West Grove, PA), washed, and counterstained with DAPI (Vector Laboratories, Burlingame, CA). Stained luminal structures were identified at low-power microscopy and counted at a \(x\)40 magnification in six random fields from the tumor.

**Measurement of Tumor Cell Proliferation (BrdUrd Incorporation).** Mice were given i.p. injections of BrdUrd 100 µg/g of body weight in 200 µl of PBS 6 h before being euthanized. Cryosections (6 µm) of tumors were digested with pepsine, denatured in 2 N H\(_2\)Cl, neutralized in borate (pH 8.5), and incubated with murine anti-BrdUrd antibody (Becton Dickinson) after permeabilization with Triton X-100. After incubation with a Cy2-conjugated goat antimouse IgG (Jackson Immunoresearch), slides were mounted in Vectashield with DAPI. Percentage of tumor-proliferating cells was calculated as: (BrdUrd\(^+\) cells)/(DAPI\(^+\) cells) \times 100.

**DNA Nick-End Labeling of Tumor Sections.** Single-cell DNA fragmentation was determined by the TUNEL method (29) using the MEBSTAIN Apoptosis kit II (Immunotech, Marseilles, France). Microvessels were costained using anti-CD31 as described above. Briefly, 6-µm cryosections were fixed in paraformaldehyde 4%, permeabilized in PBS/0.2% Triton X-100, blocked in PBS/10% BSA/0.2% Triton X-100, and incubated with terminal...
deoxynucleotidyltransferase and biotin-UTP for 1 h at 37°C for DNA nick-end labeling. Sections were additionally incubated with avidin-FITC and rat anti-mouse anti-CD31 antibody at 4°C overnight, washed, incubated with Cy3-conjugated antirat IgG, and mounted with Vectashield with DAPI. Positive DNase I-digested and negative terminal deoxynucleotidyltransferase-free controls were included. Total tumor area and apoptotic tumor area were measured using the public domain NIH Image program (version 1.62).

RESULTS

Late-Generation Terc−/− Mice Show Decreased Angiogenic Potential in a “Basement Membrane Matrix” Assay (Matrigel Assay). Alterations in tissues with high proliferative rates, such as germ cells and hematopoietic cells, are readily observed in late-generation Terc−/− mice, which show short telomeres and increased chromosomal instability (19–23). However, low cell turnover may delay tissue dysfunction because of critical telomere shortening in other Terc−/− tissues. In this regard, vascular tissue has a very low cell turnover (30, 31). To unveil possible proliferative defects in endothelial cells from late-generation Terc−/− mice, we induced rapid turnover of these cells by experimental manipulation, such as using Matrigel implants or melanoma grafts (see below).

Rapid vascularization can be elicited by Matrigel, a reconstituted basement membrane matrix from the Engelbreth-Holm-Swarm tumor (28). To study the angiogenic potential of Terc−/− mice with critically short telomeres, we injected bFGF-enriched Matrigel in the ventral s.c. tissue of wild-type, early generation (G2) and late-generation Terc−/− mice in two different genetic backgrounds (G3/G4 or G5/G6 for BL6 and MB genetic backgrounds, respectively; see “Materials and Methods”). The newly formed capillary network within the Matrigel can be quantified by measuring the hemoglobin concentration of excised implants. At day 7 after injection, late-generation Terc−/− mice in both genetic backgrounds showed a significant decrease in hemoglobin concentration compared with wild-type mice; 35% and 53% of wild-type levels for G3/G4 (BL6) and G5/G6 (MB) Terc−/− mice, respectively (these differences were significant, Student’s t test: P < 0.05; Fig. 1a, Table 1, and Fig. 1b–d for representative examples

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*wt, wild type.
Mitogens used for B and T cell stimulation.
Tumor growth is dependent on and T lymphocytes, respectively. Lymphocytes from Terc mice (Table 1; Fig. 1, showed no decrease in angiogenic potential compared with wild-type telomeres similar in length to those of wild-type controls. These mice mice in both genetic backgrounds lack telomerase activity but have mice in the MB genetic background). Early generation G2 Terc mice in both genetic backgrounds lack telomerase activity but have telomeres similar in length to those of wild-type controls. These mice showed no decrease in angiogenic potential compared with wild-type mice (Table 1; Fig. 1, a and c). Therefore, in the Terc mice studied, the failure to vascularize the Matrigel implants can be assigned to critically short telomeres but not to telomerase absence per se.

To define correlates between low and high turnover tissues in individual late-generation Terc mice, we stimulated splenocytes from Matrigel-injected animals with mitogens: LPS or con A for B and T lymphocytes, respectively. Lymphocytes from Terc mice with impaired angiogenesis (hemoglobin < 0.5 g/liter × mg Matrigel) invariably showed a significantly reduced proliferative response to both mitogens (thymidine incorporation < 60% of wild-type levels 72 h after mitogen exposure; Table 1). These results suggest that telomere length is a universal determinant of tissue replicative potential and that the latency period to dysfunction is determined by the tissue-specific proliferative rate.

**G5 Terc** Mice Show Decreased Tumor Growth Rate in a Tumorigenesis Assay (B16F10 Cells). Tumor growth is dependent on tumor vascularization by host microvasculature (32). To extend our observation with Matrigel implants to a more clinically relevant in vivo angiogenesis model, we investigated the effect of critical telomere shortening on B16F10 tumor-induced angiogenesis. B16F10 cells are murine melanoma cells that form melanotic nodules after dermal or s.c. injection (33–35). We injected 10⁵ B16F10 cells in the flanks of wild-type, G2 and G5 Terc (MB) mice and monitored nodule development with time. By day 12 after injection, the mean tumor size was 237, 176, and 35 mm³ for wild-type, G2 Terc, and G5 Terc mice, respectively (average of 18 separate injections per group; Fig. 2a; Table 2). This delayed tumor growth in G5 Terc mice was observed at all of the time points thereafter until tumor excision was performed at days 16–20 after injection (Fig. 2b).

In addition to a decreased tumor growth rate, G5 Terc mice also exhibited a lower tumor formation efficiency; 88% (16 of 18) of the B16F10 cell injections formed tumors in G5 Terc mice, whereas 100% (18 of 18) of them formed tumors in both wild-type and G2 Terc mice. Representative sets of tumors for each genotype at day 16 after injection are shown in Fig. 3; of notice, the profusion of small vessels observed in the peritumoral area of excised wild-type and G2 Terc tumors is absent in G5 Terc tumors (Fig. 3).

**Microvessel Density Is Decreased in G5 Terc** B16F10 Tumors. To investigate whether decreased tumor growth in G5 Terc mice was attributable to decreased host angiogenesis, we estimated the microvessel density in tumor cryosections by staining with an

| Table 2. B16F10 tumor growth dynamics in MB Terc mice 12 days after injection |
|-----------------------------|-----------------------------|-----------------------------|
| Tumor size (mm³)            | 237 ± 51⁵                  | 176 ± 49⁶                  |
| Microvessel density (# vessels/200 × field) | 11.3 ± 1.7⁵               | 11.4 ± 4.7⁶               |
| Tumor cell proliferation (% BrdU nuclei) | 58.8 ± 6.3⁵               | 39.0 ± 7.8⁶               |
| Tumor cell apoptosis (% tumor area) | 6.4 ± 4.2⁶                | 2.5 ± 0.7⁶                 |
| " SE.                       | ⁶ SD.                      |                             |

Fig. 3. Tumor size and peritumoral vascularization in telomerase-deficient mice. At day 12 after injection, wild-type and G2 Terc tumors appear as raised melanotic nodules of ~1 cm in diameter. In contrast, G5 Terc tumor nodules were significantly smaller (see tumor images “prior to excision”). Appearance of peritumoral s.c. tissue at the time of tumor excision is also shown for all genotypes. A network of tumor-feeding vessels is visible in the s.c. tissue of wild-type and G2 Terc tumors but has barely developed around G5 Terc tumors (see tumor images “post-excision”).
Fig. 4. Microvessel density in B16F10 tumor cryosections. a, microvessel counts in B16F10 tumor cryosections from wild-type, G2, and G5 Terc<sup>−/−</sup> mice (six mice per group). Each dot represents the average vessel number in six random ×200 fields of a representative tumor section. Bars, ±SD of six fields per tumor. b, fluorescence microscopy of tumor cryosections stained with an anti-CD31 antibody (red) and counterstained with DAPI (blue) showing microvessel morphology. In wild-type tumors, microvasculature is characterized by narrow convoluted microvessels at the tumor center and periphery. In contrast, G5 Terc<sup>−/−</sup> tumors displayed larger vessels with wide lumens, reminiscent of aged vessels (see arrows). Magnifications are ×40.

ANGIOGENESIS IN MICE WITH SHORT TELOMERES

G5 Terc<sup>−/−</sup> B16F10-induced Tumors Show Decreased Replicative Potential and Increased Apoptotic Rates. Alterations in microvessel density have been shown previously to modify B16F10 tumor growth rate by altering the balance between tumor cell proliferation and tumor cell death by apoptosis or necrosis (36). In our model, DNA synthesis measured with a BrdUrd incorporation assay was reduced in G5 Terc<sup>−/−</sup> tumors compared with wild-type and G2 Terc<sup>−/−</sup> tumors: 58.8 ± 6.3%, 59 ± 7.8%, and 43.3 ± 4.9% of BrdUrd-positive cells for wild-type, G2, and G5 Terc<sup>−/−</sup> tumors, respectively (Fig. 5a and Table 2 for quantification and Fig. 5b for examples).

Despite this difference between genotypes, the proliferating fraction in late-generation Terc<sup>−/−</sup> tumors is notably high and cannot account for the marked reduction in G5 Terc<sup>−/−</sup> tumor size. Thus, we investigated whether G5 Terc<sup>−/−</sup> tumors showed increased rates of apoptosis using the TUNEL assay to detect apoptosis in single cells. Wild-type tumors showed discrete apoptotic areas, which were clearly demarcated from surrounding viable tumor areas (Fig. 6a). In these tumors, most apoptotic foci were found at the tumor center or forming narrow rims at the subcapsular area. In contrast, apoptotic areas were significantly larger in G5 Terc<sup>−/−</sup> tumors (Fig. 6c). In particular, 6.4 ± 4.2%, 2.5 ± 0.7, and 15.7 ± 5.1% of the total tumor area was TUNEL-positive in wild-type, G2, and G5 Terc<sup>−/−</sup> tumors, respectively (Fig. 6a). Double TUNEL/anti-CD31 staining revealed that microvessels centered at viable tumor areas and were invariably missing at the apoptotic areas in both genotypes (Fig. 6, b and c; Table 2), highlighting the fact that short telomeres in the host compromise tumor graft viability. Apoptosis was never observed in tumor endothelial cells, ruling out microvessel regression as a mechanism for decreased vessel density in G5 Terc<sup>−/−</sup> tumors.

DISCUSSION

The mechanisms that modulate angiogenesis with age have not been uncovered to date (37). Telomere shortening with age has been shown previously to modify B16F10 tumor growth rate by altering the balance between tumor cell proliferation and tumor cell death by apoptosis or necrosis (36). In our model, DNA synthesis measured with a BrdUrd incorporation assay was reduced in G5 Terc<sup>−/−</sup> tumors compared with wild-type and G2 Terc<sup>−/−</sup> tumors: 58.8 ± 6.3%, 59 ± 7.8%, and 43.3 ± 4.9% of BrdUrd-positive cells for wild-type, G2, and G5 Terc<sup>−/−</sup> tumors, respectively (Fig. 5a and Table 2 for quantification and Fig. 5b for examples).

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Using optimal angiogenic doses of bFGF on a Matrigel substrate, we show here that telomerase-deficient mice with critically short telomeres in two different genetic backgrounds show a striking impairment in angiogenesis, already noticeable on visual inspection of Matrigel explants. Studies on telomerase-deficient murine keratino-
cytes and human fibroblasts pointed to known regulators of cell cycle progression and DNA damage repair, such as p53, as mediators of telomere length-dependent cell cycle arrest or increased apoptosis (38, 39). In our model, concomitant telomere shortening of vessel smooth muscle cells, fibroblasts, lymphocytes and other cell types may also condition their ability to secrete cytokines or interact with endothelial cells, contributing to the observed phenotype.

We extended our observations in the Matrigel model to a tumor graft model. B16F10 cells generate steep gradients of proangiogenic factors, such as vascular endothelial growth factor (36), responsible for the so called “angiogenic switch” that precedes tumor growth (31, 40, 41). The reduced microvessel counts on CD31-stained G5 Terc−/− tumor sections is likely to result from a decreased endothelial cell proliferation in these mice. In support of this hypothesis, we found no evidence of endothelial cell apoptosis or regression of preexisting vessels using the TUNEL assay. Furthermore, changes in microvessel morphology toward a larger, more “mature” nonproliferative phenotype were observed in G5 Terc−/− tumors. Similar morphological changes had been reported by Pili et al. (35) when comparing B16F10 tumor sections from old and young mice.

An important consequence of the decreased vascular supply to the tumor was a pronounced decrease in G5 Terc−/− tumor growth rate. In fact, G5 Terc−/− tumors were on average only 15% the size of wild-type or G2 Terc−/− tumors. Using an experimental design very similar to ours, a decrease of B16F10 tumor size of the same magnitude was reported when comparing 28-month-old mice to 3-month-old mice (34, 35). Therefore, telomere shortening in young age, late-generation Terc−/− mice leads to an angiogenic response that can be grossly compared with that of aged wild-type mice. It is important to note that B16F10 cells are weakly antigenic, and their growth rate is mainly determined by host local factors such as tumor-induced host angiogenesis. In this regard, the growth rate of more antigenic tumors
could also be influenced by other age-related changes in the host such as immunosenescence (4).

This work also sheds light on the debated role of vascular density as a predictor of tumor aggressiveness and clinical prognosis. Tumor microvessel count has been advocated in the past as an independent prognostic factor in human cancer (42, 43). However, the heterogeneity of vascular density in different histological tumor types (44, 45), the lack of a clear correlation between tumor vascularity and tumor endothelial cell proliferation (46), and the presence of immature vessels and mosaic vessels in many tumors (44) have precluded a consensus on this issue. We show here that for B16F10 cells tumor microvessel density seems to be a predictor of tumor behavior. However, we cannot rule out that other factors besides angiogenesis may have contributed to the decreased tumor growth in late-generation telomerase-deficient mice. Indeed, telomere shortening affects all tissues of this mouse model, and a deficiency in fibroblast- or macrophage-secreted factors or in hematopoietic precursors may also be influencing tumor development in these mice.

Our findings provide new insights on endothelial cell biology that are useful for designing new therapies. The decreased angiogenesis potential of mice with critically short telomeres also suggests a role for telomere length in some human age-associated vascular diseases such as arteriosclerosis and delayed wound healing, which might benefit from therapies aimed to maintain or restore telomere length of vascular tissues. On the other hand, telomeres of endothelial cells may be viewed as potential targets in angiogenesis-dependent diseases, such as cancer and inflammatory diseases. A tumor therapy based on eliciting telomere shortening (i.e., telomerase inhibition) would contribute to tumor size reduction not only by a cytolytic effect (47) but also by slowing or preventing angiogenesis when a critically short telomere length is reached. Furthermore, antitelomerase therapy of tumor endothelial cells may potentiate other therapeutic modalities. In particular, telomere shortening has been shown to increase sensitivity to radiation in vivo (48), and the combination of antiangiogenic therapies with ionizing radiation has been shown to be synergistic in reducing tumor size (49, 50). These observations, put together with our finding of reduced angiogenic potential with telomere shortening, suggest that combinations of antitelomerase and antiangiogenic therapies may act as potent modifiers of radiation sensitivity.

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