Characterization of primitive hematopoietic cells from patients with dyskeratosis congenita

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Dyskeratosis congenita (DC) is an inherited bone marrow (BM) failure syndrome associated with mutations in telomerase genes and the acquisition of shortened telomeres in blood cells. To investigate the basis of the compromised hematopoiesis seen in DC, we analyzed cells from granulocyte colony-stimulating factor mobilized peripheral blood (mPB) collections from 5 members of a family with autosomal dominant DC with a hTERC mutation. Premobilization BM samples were hypocellular, and percentages of CD34+ cells in marrow and mPB collections were significantly below values for age-matched controls in 4 DC subjects. Directly clonogenic cells, although present at normal frequencies within the CD34+ subset, were therefore absolutely decreased. In contrast, even the frequency of long-term culture-initiating cells within the CD34+ DC mPB cells was decreased, and the telomere lengths of these cells were also markedly reduced. Nevertheless, the different lineages of mature cells were produced in normal numbers in vitro. These results suggest that marrow failure in DC is caused by a reduction in the ability of hematopoietic stem cells to sustain their numbers due to telomere impairment rather than a qualitative defect in their commitment to specific lineages or in the ability of their lineage-restricted progeny to execute normal differentiation programs. (Blood. 2008; 111:4523-4531)

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Introduction

Dyskeratosis congenita (DC) is an inherited multisystem disorder of premature aging characterized by early bone marrow (BM) failure.1,2 The 2 major forms of this disease, X-linked and autosomal dominant (AD), are associated with mutations in specific genes that are components of the telomerase complex. In the X-linked form, DKC1 gene mutations have been found that affect dyskerin expression, a small nucleolar ribonucleoprotein involved in ribosomal RNA processing that is also involved in the processing and stabilization of the RNA component of telomerase, hTERC.3,4 In AD DC, several different mutations have been identified in hTERC.5,6 Relevant to the current study, a large multigenerational AD DC family was identified in Iowa whose affected members have a deletion of the last 74 base pairs of the 3' end of one hTERC allele.6 Recently, mutations in the reverse-transcriptase component of telomerase hTERT have also been found in at least one AD DC family.7 An autosomal recessive form of this disorder also exists, with mutations of NOP-10 being identified.8 Of note, homozygous hTERT mutations have also been found in 2 distinct consanguineous families, further accounting for other incompletely defined cases of autosomal recessive DC.9 Somatic cells from DC patients have markedly shortened telomeres, which is presumed to be a consequence of their telomerase deficiency.1,4,10,11 The clinical triad of DC consists of abnormal skin pigmentation, nail dystrophy, and mucosal leukoplakia.12 Morbidity is often associated with the development of cytopenia, and most X-linked DC patients do not survive past 30 years of age due to complications of marrow aplasia.13 It has been suggested that X-linked disease has a more severe phenotype than the AD form.14 However, the primary determinant of disease onset and severity does not appear to be telomerase levels per se but telomere length at birth, and ongoing observations of the multigeneration family with AD DC from Iowa support a model of disease anticipation15 in which later-generation subjects with hTERC mutations manifest clinical symptoms earlier than their parents.

Telomere length and telomerase play an essential, although not fully understood, role in normal hematopoiesis, and telomere length is clearly involved in the pathogenesis of DC.16-19 Previous studies on the Iowa AD DC family showed that haploinsufficiency of hTERC leads to telomere shortening of hematopoietic-derived cells compared with age-matched controls.20 While some subjects had peripheral cytopenias, nearly all had immune abnormalities, including marked B lymphopenia, decreased immunoglobulin M (IgM) levels, and overexpression of senescent markers on T cells.21 Of note, short telomeres were also observed in noncarrier siblings of those with hTERC mutations, although none had clinical or laboratory evidence of DC. This observation suggests that only below a certain threshold does telomere length affect the function of hematopoietic-derived cells. Shortened telomeres have been reported in other BM failure disorders, including Shwachman-Diamond syndrome (SDS)22 and aplastic anemia,23 while mutations and polymorphisms within genes encoding telomerase components have been noted in “idiopathic aplastic anemia.”24,25


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However, it remains unknown whether the degree of cytopenia or onset of BM aplasia correlates with telomere length in hematopoietic cells or may be a function of defects in the microenvironment. It is also unclear whether the defects observed in BM function in DC are a derivative of a compromised hematopoietic stem cell (HSC) population, and if so, whether this is primarily due to qualitative or quantitative perturbations of this compartment. These questions are of major significance to understanding the hematopoietic defect in DC and will also be important for developing future strategies to treat DC patients effectively. To this end, we undertook studies to phenotypically and functionally characterize the circulating CD34+ cells that were mobilized by granulocyte colony-stimulating factor (G-CSF) administration to DC patients.

Methods

Subjects and controls

All DC subjects enrolled on these studies signed informed consents that were approved by the University of Iowa Children’s Hospital Institutional Review Board and in accordance with the Declaration of Helsinki. The diagnosis of AD DC was confirmed by mutational analysis, demonstrating a 74-base pair deletion in a single hTERC allele, as previously described. Eligibility requirements at the time of mobilized peripheral blood (mPB) collections allowed for the presence of mild cytopenias in DC subjects. Minimal values were as follows: hemoglobin level higher than 10 g/dL, absolute neutrophil count (ANC) higher than 1000/mm3, and a platelet count higher than 50 000/mm3. In addition, precollection BM aspirations were analyzed to ensure subjects had no evidence of leukemic transformation or myelodysplasia. Control cells, mPB, and BM were obtained from clinically discarded samples at the University of Iowa Hematopoietic Stem Cell Processing Laboratory that had been isolated from healthy donors and were no longer required.

BM analysis

BM aspirates were collected and the CD34+ cell content was measured according to the International Society of Hematotherapy and Graft Engineering guidelines. Hematocrit and eosin staining was used to assess morphology and cellularity. BM aspirates were also sent for standard cytogenetics, fluorescent in situ hybridization (FISH) analyses, and diepoxybutane (DEB)–induced chromosome breakage studies.

Collection of mPB cells

Prior to collecting the mPB cells by leukapheresis, all subjects received 10 μg/kg G-CSF (Amgen, Thousand Oaks, CA) subcutaneously daily for 5 days, with the last 2 injections occurring in the morning just prior to leukapheresis. DC subjects were required to weigh at least 40 kg such that peripheral intravenous catheters were of sufficient gauge to allow apheresis collections to be undertaken. The collection protocol aimed to collect a minimum of 2 × 10^6 CD34+ cells/kg. Complete blood counts (CBCs) with differential and CD34+ analyses from peripheral blood (PB) were obtained prior to harvesting, but the collection was allowed to proceed independent of the pre-apheresis CD34+ counts. Peripheral intravenous catheters were placed bilaterally into the antecubital veins, and 5 blood volume collections (3.5-6 liters) were performed over 3 to 4 hours in the University of Iowa Hospitals and Clinics DeGowin Blood Center on 2 consecutive days using a Cobe Spectra instrument (Gambro BCT, Lakewood, CO). Total nucleated cell counts, mononuclear cell counts, and CD34+ cell counts were obtained on each apheresis collection. Each collection was cryopreserved using a rate controlled freezer (Custom Biogenics Systems, Shelby Township, MI). Ten percent of each collection was separately cryopreserved to allow laboratory studies to be performed.

Immunophenotyping

Immunophenotyping of CD34+ cells from leukaemia collections was performed on fresh and frozen/thawed products from DC subjects and a range of age-matched controls. Briefly, mononuclear cells were stained with a combination of fluorescein- or phycoerythrin-conjugated antibodies to CD34, CD33, CD38, CD19, CD7, CD71, and CD41 (Becton Dickinson, San Jose, CA) as previously described. Cells were analyzed on a FACScan flow cyrometer (Becton Dickinson) using the International Society of Hematotherapy and Graft Engineering gating strategies, and data analysis used Cellquest V3.2 software (Becton Dickinson).

In vitro assays

Populations enriched in CD34+ cells were isolated immunomagnetically (Easy Sep; StemCell Technologies, Vancouver BC) from thawed DC and control cells according to the manufacturer’s instructions (purity of DC samples = 2%-38%; purity of normal controls 5%-82%). To determine the frequency of colony-forming cells (CFCs) within the CD34+ compartment of these samples, aliquots were then plated in standard MethoCult H4434 (StemCell Technologies) that contains interleukin-3 (IL-3), IL-6, steel factor (SF), and erythropoietin (EPO). Erythroid colonies (from erythroid burst-forming units [BFU-E]), granulopoietic colonies (from granulocyte-macrophage colony-forming units [CFU-GMs]), and multilineage colonies (from granulocyte, erythrocyte, monocyte, macrophage colony-forming units [CFU-GEMMs]) were scored by direct visualization using an inverted microscope (40×) 14 days later using standard criteria. These values were then added together to obtain total CFC counts and expressed per 10^6 actual CD34+ cells plated. The average number and types of mature cells in the colonies generated in these assays was determined by harvesting the cells from diluted 14-day-old cultures and then performing cell counts and fluorescence-activated cell sorting (FACS) analyses (using anti–glycoporphin A, anti-CD15/66b, anti-CD33, and anti-CD41a antibodies). Long-term culture-initiating cell (LTC-IC) frequencies were determined as previously described. In brief, a defined number of CD34+ test cells were cocultured with irradiated mouse fibroblasts engineered to make human IL-3, G-CSF, and SF for 6 weeks at 37°C, and then the total number of CFCs present was determined on the harvested cells. LTC-IC numbers were then calculated assuming each LTC-IC will have produced an average of 25 CFCs detectable at that time. Photomicrographs of colonies used a Canon EOS digital Rebel XT camera (Tokyo, Japan) mounted on a Zeiss microscope model 475638 (Carl Zeiss, Heidelberg, Germany), with magnification of objective lens 2.5×08 and eyepiece magnification of 12.5×/20W.

Telomere length measurements: flow-FISH, Q-FISH, and STELA

Telomere length measurements using flow-fluorescence in situ hybridization (flow-FISH) was performed as described. Briefly, white blood cells (WBCs) were isolated by osmotic lysis of erythrocytes in whole blood samples using NH4Cl. The WBCs were then mixed with bovine thymocytes of known telomere length (internal control), denatured in formamide at 87°C, and hybridized with a fluorescein-conjugated (CCCTAA)3 peptide nucleic acid (PNA) probe specific for telomere repeats and counterstained with LDS751 DNA dye. The fluorescence intensity in WBCs, granulocytes, and total lymphocytes relative to internal control cells was measured on a FACSCalibur instrument (Becton Dickinson) to calculate telomere length.

Quantitative fluorescence in situ hybridization (Q-FISH) with Cy3–labeled (CCCTAA)3 peptide nucleic acid (PNA) and analysis of telomere length from digital images were performed as described. Briefly, Cy3–labeled PNA probe (CCCTAA)3 specific for mammalian telomeres (Applied Biosystems, Foster City, CA) was hybridized to metaphase chromosomes obtained from 4-day-old suspension cultures initiated with CD34+ mPB cells cultured in StemSpan (StemCell Technologies) containing IL-3, IL-6, SF, and EPO. To remove nonhybridized PNA, slides were washed twice for 15 minutes each time with wash solution I (0.1 M Tris, 0.15 M NaCl, 0.08% Tween 20, pH = 7.0–7.5). Slides were counterstained with the DNA dye DAPI and examined using a Zeiss Axioplan II fluorescence microscope (Carl Zeiss). For each DC
sample, 15 individual metaphase nuclei were analyzed, and the mean fluorescence intensity (MFI) was correlated to telomere length as measured relative to plasmid standards with fixed numbers of telomere repeats. The fluorescent signal was then quantified and analyzed for Q-FISH image analysis (Flintbox, http://www.flintbox.com).

Single telomere analysis (STELA) was conducted as described previously. Briefly, enriched CD34+ mPB cells were added directly into tubes containing 1 × SSC and 1 × 10^5 SF9 insect cells. Since only a small number of enriched CD34+ cells were tested, inclusion of SF9 cells acted as both a cellular and genomic carrier. DNA was extracted from enriched populations by standard proteinase K, RNase A, and phenol/chloroform protocols. DNA was ligated at 35°C for 12 hours with 0.001 μM telotere linker 806 that anneals to the telomeric 3’ G-rich overhang and ligates directly to the 5’ end of the telomere. DNA was diluted to 50 genome equivalents per microliter, based on cell numbers obtained from sorting and added to a polymerase chain reaction (PCR) reaction containing oligonucleotides 800 and 810, specific for the Xyp subtelomeric region and a sequence on the linker 806, respectively. A total of 24 rounds of amplification were undertaken, and samples were resolved on 0.7% LE agarose, Southern blotted, and hybridized to a probe specific to the XpYp subtelomeric region (generated by PCR using primers 800 and 801).

### Results

#### Clinical characteristics and BM findings on DC subjects

Initial clinical data on subjects identified as having AD DC were previously published, and laboratory studies on these subjects have also been recently reported. Over the past 6 years, subjects have been followed on a yearly basis, and CBCs obtained over that period have shown a slight decline in platelet counts in several individuals, specifically those in the third generation (UPNs 7 and 9). Due to the possibility of progressive BM failure, subjects were enrolled. All had clinical and laboratory features of DC, including erythrocyte macrocytosis and mild cytopenia (Table 1).

Bone marrow aspirations were performed prior to apheresis collections and showed an overall decrease in BM cellularity in all DC subjects. This was most striking in the youngest third-generation subjects (UPNs 7 and 9, Table 2). However, there was no evidence of dyspoiesis, myelodysplasia, or leukemia, and the myeloid-erythroid ratios were within the normal range, as were the percentages of hematopoietic intermediates, although the percentages of lymphocytes in the BM smears were increased in 3 of the 5 cases. Of note, decreased numbers of megakaryocytes were observed in all BM except that from UPN 5. Strikingly, the percentage of CD34+ cells in the BM aspirates was lowest in the 2 third-generation subjects (0.05% and 0.16% compared with 0.5%-1% in the healthy controls). Cytogenetic and FISH analyses of the BM cells showed no gross abnormalities, although the possibility of unresolved mutations would not be precluded by these analyses. No increase in chromatin breakage was found in DEB-supplemented cultures of DC lymphocytes suggesting no gross defects in DNA repair (data not shown).

#### mPB collections

All subjects tolerated 5 daily subcutaneous injections of G-CSF without significant toxicity and, while mild discomfort was reported, none required analgesia for myalgias or bone pain. HSC collections proceeded without incident and, per routine, oral calcium supplements were administered throughout to prevent hypocalcemia. Hemograms were obtained prior to initial collections and, as noted in Table 3, the post–G-CSF/pre-apheresis ANC and total WBC count increased only moderately in all DC subjects except UPN 2, where a more typical increase was observed (WBC count = 38 900/mm^3). Following 2 days of mPB cell collections, hemoglobin and WBC counts remained fairly close to preapheresis values. In contrast, platelet counts were markedly decreased from baseline values. Of note, no subject required transfusion support.

<table>
<thead>
<tr>
<th>Subject</th>
<th>UPN 2</th>
<th>UPN 5</th>
<th>UPN 6</th>
<th>UPN 7</th>
<th>UPN 9</th>
<th>Normal range</th>
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<td>Clinical findings</td>
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<td>S,L,N</td>
<td>S,L,N</td>
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<td>N</td>
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<td>Hemoglobin, g/dL</td>
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<td>13.7</td>
<td>13.4</td>
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<td>95</td>
<td>104</td>
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<td>92</td>
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<tr>
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<td>49</td>
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<tr>
<td>WBC count, ×10^9/mm^3</td>
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<td>3.3</td>
<td>3.4</td>
<td>3.4</td>
<td>4.7</td>
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<td>ANC, ×10^3/mm^3</td>
<td>1.426</td>
<td>1.400</td>
<td>1.360</td>
<td>1.894</td>
<td>2.350</td>
<td>1.550-6.100</td>
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</tbody>
</table>

MCV indicates mean corpuscular volume; WBC, white blood cell; ANC, absolute neutrophil count; S, skin dyspigmentation; L, leukoplakia; and N, nail dystrophy.

CTRL indicates control; ep, erythrocyte precursor; pm, promyelocytes; my, myelocytes; mmy, metamyelocytes; segs, segmented neutrophils; baso, basophils; mono, monocytes; pro-mono, promonocytes; and eos, eosinophils.

Markedly hypocellular relative to age-matched controls.
nor had any bleeding complications. When subjects were seen for routine follow up 6 months later, platelet counts had returned to preapheresis levels (data not shown). Although the target was to collect 1 to $2 \times 10^7$ CD34+ cells, based partially upon subject weight and the number of cells routinely used in transplantation protocols, this number was met for only 2 subjects (UPNs 2 and 5). Only $3.4 \times 10^7$ CD34+ cells (~0.5 to $10^6$ CD34+ cells/kg) were collected from the other second-generation subject, UPN 6. This subject had experienced significant thrombocytopenia over the previous 10 years and had a more pronounced phenotype than the other second-generation subjects. Yet the lowest yield was in the third-generation subject, UPN 7, from whom only $3.2 \times 10^7$ CD34+ cells were collected. Of note, this subject also had the lowest percentage of CD34+ cells in both the mPB product and in the BM. She also had a long-standing history of thrombocytopenia. The youngest subject, UPN 9, had a several year history of mild thrombocytopenia, and the collection yielded a total of 9.7 to $10^7$ CD34+ cells.

Phenotypic characterization of DC cells

Immunophenotyping experiments carried out on mPB cells from DC subjects and age-matched controls showed that the representation of subsets of CD34+ cells coexpressing erythroid (CD71), B- and T-lymphoid (CD19 and CD7), megakaryocytic (CD41), and granulopoietic (CD33) markers, or that had a very primitive phenotype (were CD38−) was similar to that seen in age-matched controls and, in almost all cases, values for DC subjects fell within the control range. The only exception was for UPN 9, where the percentage of CD34+CD38− cells was higher than normal. A similar finding was also noted in the BM sample from this patient (data not shown).

Functional characterization of primitive DC cells

CFC assays performed on CD34+ enriched mPB cells from all 5 DC subjects revealed the frequencies of granulopoietic (CFU-GM), erythroid (CFU-E + BFU-E), and multilineage (CFU-GEMM) progenitors to be similar to the frequencies of these progenitors in comparable populations isolated from mPB harvests from 5 healthy donors. Figure 1A shows the results for the total CFC numbers per $10^9$ CD34+ cells. Comparison of the total size and distribution of different types of colonies produced by the normal and DC cells in these assays indicated these were also indistinguishable (Figure 1B, data not shown), and this was confirmed by more detailed immunophenotyping analyses of the different lineages present within the pooled colonies from the CFC assay (number of nucleated glycoporphin A+ cells generated in CFC assays per $10^3$ CD34+ mPB cells plated was 33, 34, 119, 210, and 359 and corresponding values for DC assays were 17, 48, 92, 146, and 153; $P > .05$). Parallel values for CD16 and/or CD66b+ cells were 2, 4, 5, 5, and 6 for the normal cells and 1, 6, 7, 14, and 125 for the DC cells ($P > .05$). However, given the markedly reduced frequency of CD34+ cells in the BM of the DC subjects and the associated reduced absolute numbers of CD34+ cells that could be collected from them, it can be inferred that the total CFC numbers in the DC subjects were significantly reduced.

LTC-IC assays performed on the same 5 DC and 5 control mPB samples revealed an overall 10-fold reduction ($P < .05$) in the

<table>
<thead>
<tr>
<th>Name</th>
<th>UPN 2</th>
<th>UPN 5</th>
<th>UPN 6</th>
<th>UPN 7</th>
<th>UPN 9</th>
</tr>
</thead>
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<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hgb level, g/dL</td>
<td>12.8</td>
<td>13.8</td>
<td>13.2</td>
<td>12.7</td>
<td>12.1</td>
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<tr>
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<td>177</td>
<td>90</td>
<td>39</td>
<td>107</td>
</tr>
<tr>
<td>WBC count, $\times 10^9$/mm$^3$</td>
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<td>12.1</td>
<td>9.6</td>
<td>5.9</td>
<td>14.8</td>
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<td>ALC, $\times 10^9$/mm$^3$</td>
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<td>1.9</td>
<td>1.3</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>ANC, $\times 10^9$/mm$^3$</td>
<td>33.6</td>
<td>9.1</td>
<td>7.5</td>
<td>3.9</td>
<td>11.9</td>
</tr>
</tbody>
</table>

| Postcollection CBC | | | | | |
| Hgb level, g/dL | 11.2 | 13.1 | 11.6 | 10.7 | 10.7 |
| Platelet count, $\times 10^9$/mm$^3$ | 40 | 69 | 29 | 11 | 48 |
| WBC count, $\times 10^9$/mm$^3$ | 29.2 | 16.8 | 12.1 | 5.8 | 10.1 |
| ALC, $\times 10^9$/mm$^3$ | 0.7 | 1.1 | 0.8 | 0.7 | 1.2 |
| ANC, $\times 10^9$/mm$^3$ | 26.4 | 14.6 | 10.1 | 4.8 | 7.9 |

Hgb indicates hemoglobin (g/dL); Plt, platelet count; WBC, white blood cell; ALC, absolute lymphocyte count; ANC, absolute neutrophil count; TNCs, total nucleated cells; and MNCs, mononuclear cells.

*Precollection CBC values were obtained just prior to the first apheresis, and postcollection CBC values were obtained 30 minutes following the first apheresis.

†Percentage CD34+ represents average values from both collections.

‡Represents number of cells collected from first collection.

§Average number of cells collected from healthy HSC apheresis volunteers (n = 11) at University of Iowa DeGowin Blood Center.

Values are expressed as percentage of coexpression on CD34+ mPB cells.
frequency of LTC-ICs within the CD34+ subset of DC cells with DC values ranging from 2- to 40-fold below the minimum normal value measured (Figure 1C). Given the further reduction in the total number of CD34+ cells in the DC subjects, the absolute decrease in their LTC-IC compartment would be very profound.

Telomere length analysis of DC cells

Telomere length analysis was performed by flow-FISH on PB samples over a period of a year and a half from 2 selected second- and third-generation AD DC subjects (UPNs 5 and 9, respectively). Both subjects showed telomere lengths below the first percentile of the normal distribution irrespective of age (Figure 2). Measurements at the first time point were performed on both PB and apheresis samples with similar results (data not shown). Telomeres were shorter in lymphocytes than in granulocytes (UPN 5: 4.6 to 3.7 Kb for lymphocytes vs 5.5 to 4.3 Kb for granulocytes; UPN 9: 3.6 to 3.1 Kb for lymphocytes vs 3.9 to 3.5 Kb for granulocytes), and these values appeared to decrease over the course of the study period, although this trend did not reach significance. These results provide further support for a mechanism of disease anticipation in this AD DC family where the symptoms and disease phenotype of the third-generation subject (UPN 9) were associated with earlier disease thus serves as an excellent model to investigate the effects of telomerase deficiency on different cellular functions, and in particular, those of the hematopoietic system. A major question is whether increased telomere shortening represents a causal link between HSC turnover, replicative senescence, and/or the induction of genetic instability in DC hematopoietic cells and possibly other acquired BM failure disorders. Furthermore, one could hypothesize that BM failure in DC reflects a qualitative impairment

Discussion

DC is a premature aging disorder of telomere attrition characterized by both progressive telomere shortening and BM failure. This disease thus serves as an excellent model to investigate the effects of telomerase deficiency on different cellular functions, and in particular, those of the hematopoietic system. A major question is whether increased telomere shortening represents a causal link between HSC turnover, replicative senescence, and/or the induction of genetic instability in DC hematopoietic cells and possibly other acquired BM failure disorders. Furthermore, one could hypothesize that BM failure in DC reflects a qualitative impairment
of the normal ability of HSCs and their progeny to execute a variety of differentiation programs and/or an altered ability to sustain HSC numbers. To begin to address these questions, we undertook a characterization of the CD34+/H11001 cells present in the BM and G-CSF–mobilized product harvested from a family of DC patients.

Two lines of evidence were obtained to indicate that the HSC compartment of DC subjects is profoundly reduced, even before the onset of severe symptoms of BM failure or transformation. The first was the reduced frequency of CD34+/H11001 cells in the preapheresis hypocellular BM aspirates studied and the poor yields of CD34+/H11001 cells in the G-CSF–mobilized products collected from the DC patients. These data are similar to that reported for Fanconi anemia and aplastic anemia patients, where BM CD34+/H11001 cell frequencies were also significantly lower than in healthy donors. The reduced absolute numbers of CD34+/H11001 cells in vivo would also mean that the absolute numbers of CFCs were correspondingly reduced in DC patients, even though their frequency in the CD34+ subset was not different from normal. The second result indicative of a reduced HSC compartment in DC patients was the further dramatic reduction within the mobilized CD34+ cells of more primitive cells identified functionally as LTC-ICs. LTC-ICs represent a cell type that is closely related to HSCs. Evidence of a more pronounced deficiency of the most primitive hematopoietic cells was also noted in other BM failure syndromes, including idiopathic aplastic anemia and paroxysmal nocturnal hemoglobinuria.

At the same time, we obtained data indicating that the differentiation program of primitive hematopoietic cells from DC subjects is not grossly perturbed. This included the finding that differentiating CD34+ cells coexpressing various lineage markers were represented at normal frequencies within the total CD34+ population isolated directly from patients. Similarly, more primitive progenitors detected by their clonogenic activity were also present within the CD34+ cells at normal frequencies and were found to display the same proliferative and myeloid differentiation activity in vitro as their counterparts in mPB samples obtained from healthy donors.

Mounting evidence suggests a relationship between HSC turnover and the shortening of WBC telomeres (particularly granulocytes that have a very short lifespan), during both normal ontogeny and after transplantation, and may even limit HSC regenerative ability. It is therefore likely that telomere shortening also plays a causal role in DC, BM failure disorders, and different forms of aplastic anemia, all of which are associated with shorter telomeres in both mature WBCs and in their more primitive precursors. In the present study, telomere lengths in PB and apheresis samples were found to be shorter than normal in both patients who were assessed at the time of the aphereses. Therefore, it is inviting to speculate that this may reflect a higher turnover rate of the HSC population in DC patients. However, the best human HSC purification strategies applied to normal BM or mPB yield very limited numbers of cells and even these are not pure suspensions of HSCs.
Therefore in DC samples where the HSC compartment appears markedly reduced, it is not possible to analyze their telomere lengths directly. Nevertheless we were able to perform telomere measurements by Q-FISH and STELA in CD34<sup>+</sup>/H11001 mPB cells after a very short period in culture and confirmed that these also have reduced telomere lengths. Furthermore, we detected telomere free ends in metaphases from CD34<sup>+</sup>/H11001 hematopoietic progenitors (Figure 3); however, no end to end fusions were found, suggesting at least partial integrity of telomere function in the CD34<sup>+</sup> cells that were examined. Further proliferation of these cells would be expected to lead to further telomere loss followed by telomere dysfunction and growth arrest due to a DNA damage response. These findings thus provide support for the possibility that BM failure in DC subjects may be a consequence of HSCs and progenitor cells entering a state of proliferative arrest or death in third-generation subjects due to an accumulated shortening of their telomeres over multiple generations.

In addition to the proposed intrinsic effect of telomere length and levels of telomerase on replicative exhaustion of the HSC pool in vivo, the stroma of DC subjects may also be compromised. This hypothesis has been suggested as a possible cause of BM failure in SDS. Not only were there lower numbers of CD34<sup>+</sup> cells in the BM of SDS patients, but SDS stromal cells failed to support hematopoiesis in vitro. More recently, using a telomerase knockout mouse model, additional data have been obtained to indicate that telomere dysfunction may extrinsically impair HSC function by affecting mesenchymal and stromal cell functions.

From a clinical perspective, it has been difficult to predict either the time of onset or the degree of BM failure in DC, and clear differences exist based upon the inheritance pattern of DC. This is of critical importance because unlike other forms of aplastic anemia, DC is associated with a rather poor outcome with respect to treatment with allogeneic HSC-containing transplants. Treatment with
Our findings may be applicable to all forms of DC. Functionally impaired in their ability to differentiate into specific lineages, although it may be difficult to collect sufficient HSCs to allow permanent repopulation unless coupled with a method for expanding their genetically corrected HSCs ex vivo. However, since DC is a heterogeneous disorder and the phenotype is influenced by the specific gene mutation, further investigations are warranted to establish whether our findings may be applicable to all forms of DC.

In summary, our study provides new evidence that telomerase insufficiency and short telomeres at least initially cause a quantitative change in HSC numbers rather than a qualitative change in their ability to differentiate. These findings point to the need for new approaches to study the effects of telomere shortening directly in HSCs and support the concept of collecting autologous HSCs early in the course of disease for potential future use in marrow rescue strategies.

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References

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