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Erosion of telomeric single-stranded overhang in patients with aplastic anaemia carrying telomerase complex mutations

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Abstract

Background—Loss-of-function mutations in telomerase complex genes reduce telomerase activity and shorten overall telomere length in leucocytes, and they can clinically manifest as bone marrow failure (aplastic anaemia and dyskeratosis congenita) and familial pulmonary fibrosis. Telomeres are constituted of double-stranded tandem TTAGGG repeats followed by a 3' G-rich single-stranded overhang, a crucial telomeric structural component responsible for the t-loop formation.

Materials and methods—We investigated the length of telomeric overhangs in 25 healthy individuals from 0 to 76 years of age, 16 patients with aplastic anaemia, and 13 immediate relatives using a non-denaturing in-gel method and the telomere-oligonucleotide ligation assay.

Results—Telomeric overhang lengths were constant from birth to eighth decade of life in healthy subjects, in contrast to overall telomere length, which shortened with ageing. Most patients with marrow failure and a telomerase gene mutation showed marked erosion of telomeric overhang associated with critically short telomeres; in other aplastic patients with normal genotypes, normal overall telomere lengths and who responded to immunosuppressive therapy, telomeric overhangs were maintained.

Conclusions—Telomeric overhang erosion does not participate in physiological ageing but support a role for eroded telomeric overhangs and abnormal telomere structure in pathological shortening of telomeres, especially caused by loss-of-function telomerase mutations. Disrupted telomere structure caused by short telomeric overhangs may contribute to the mechanisms of abnormal haematopoietic compartment senescence and chromosomal instability in human bone marrow failure.

Keywords

Aplastic anaemia; dyskeratosis congenita; mutation; telomerase; telomere; telomeric overhang

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Introduction

Acquired aplastic anaemia (AA) is a human disease characterized by low blood cell counts and a hypocellular bone marrow [1]. If not adequately treated by either haematopoietic stem-cell transplant or intensive immunosuppressive therapy, severe AA is often fatal because of invasive infections or severe bleeding. Some patients with acquired AA share genetic characteristics with the constitutional syndrome dyskeratosis congenita (DKC) and have markedly shortened telomeres of leucocytes, a finding associated with higher rates of clinical relapse and evolution to myelodysplasia and acute myeloid leukaemia in acquired aplastic anaemia [2,3].

Telomeres are ribonucleoproteins consisting of double-stranded TTAGGG tandem repeats; telomeres are located at the ends of chromosomes and protect them from recombination, end-to-end fusion and recognition as damaged DNA [4]. In somatic mature cells, telomeres are gradually lost with replication, as a result of DNA polymerase's inability to fully synthesize the 5' end of DNA lagging-strand [5] eventually leading to critically short telomeres, proliferation arrest, senescence and chromosomal instability. To counter telomere attrition in highly proliferative cells, including haematopoietic stem and progenitor cells, telomeres are catalytically elongated by addition of hexameric TTAGGG repeats to the 3' end of telomeric DNA by telomerase, a specialized reverse transcriptase composed of the enzyme, telomerase reverse transcriptase (TERT), its integral RNA template (TERC), and associated proteins [6].

Patients with DKC show marked telomere shortening. DKC is a constitutional form of bone marrow failure clinically associated with a mucocutaneous triad: nail dystrophy, reticular hyperpigmentation and leukoplakia, as well as lung fibrosis, oesophageal stricture, liver cirrhosis and increased risk of cancer [7]. Mutations in the gene encoding dyskerin (*DKC1*), which associates with telomerase, cause X-linked DKC [8]; mutations in *TERC*, *TERT* and *TINF2* (encoding the telomere-protecting protein TIN2) are aetiological in autosomal dominant DKC [2,9,10]; autosomal recessive DKC results from mutations in *TERT*, *NHP2* and *NOPI0* [2,11–13]. Mutations in *TERC* and *TERT* also are found in patients with apparently acquired AA [14–16] who lack the classical physical abnormalities typical of dyskeratosis congenita. These mutations associate with insufficient telomerase activity in haematopoietic cells, markedly short overall telomere length, cellular senescence and consequent poor haematopoietic function [2,16,17].

It has been suggested that telomeric single-stranded overhang plays a critical role in triggering cell senescence, based on observations of certain human cell lines [18]. Telomeric overhangs are the 3' G-rich single-stranded DNA following telomere double-stranded tandem repeats. Overhangs are critical for telomere function because they form the t-loop; the overhang invades the telomeric double helix, remodelling the DNA at the ends of chromosomes into a circle (Fig. 1) [19], a process important for chromosomal stability as well as for the extension of telomeres by telomerase [4]. From initial experiments, erosion of telomeric overhang was inferred to be a specific molecular alteration of telomeres, triggering cell senescence [18]. These results and their interpretation were subsequently challenged by the finding that normal human fibroblasts maintain overhang length with senescence despite

overall telomere shortening. Thus, the role of 3' G-rich overhang attrition in triggering cell senescence remains controversial.

In this study, we first investigated whether, analogous to overall telomere length, telomeric overhangs also shortened with physiological ageing, and then examined whether telomeric overhang erosion associated with telomerase mutations and telomere shortening in bone marrow failure.

Materials and methods

Patients and controls

Peripheral blood samples of ten AA patients carrying telomerase mutations (six had a *TERT* mutation and four had a *TERC* mutation), their mutation-carrier and non-carrier relatives, five patients with acquired AA with normal telomere lengths and robust response to immunosuppressive therapy (Table 1), and 25 healthy controls varying in age from 0 to 76 years were collected and DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen Sciences, Germantown, MD, USA) from total peripheral blood leucocytes isolated by red cell lysis. The diagnosis of aplastic anaemia was based on the bone marrow and blood-count criteria of the International Agranulocytosis and Aplastic Anemia Study [20]. Severe AA was defined as bone marrow cellularity of less than 30% and severe pancytopenia with at least two of the following peripheral blood count criteria: (i) absolute neutrophil count less than $0.5 \times 10^9 \text{ L}^{-1}$; (ii) absolute reticulocyte count less than $60 \times 10^9 \text{ L}^{-1}$; and (iii) platelet count less than $20 \times 10^9 \text{ L}^{-1}$. All other AA cases that did not fulfil the severity criteria were classified as moderate AA. Bone marrow biopsy studies did not identify dysplastic changes in any patient at diagnosis. Cytogenetic analyses were performed on bone marrow aspirate samples for all patients at presentation and resulted negative for chromosomal abnormalities. Patient A developed an isochromosome +i(1)(q10) 15 years after diagnosis (Table 1). All patients tested negative for Fanconi anaemia on *in vitro* exposure of lymphocytes to diepoxybutane and to mitomycin C. Patients, relatives or their guardians provided written informed consent for genetic testing, according to protocols approved by the institutional review board of the National Heart, Lung, and Blood Institute.

Telomere length measurement

Telomere length of peripheral blood leucocytes was measured by telomeric restriction fragment (TRF) length with a 50-box grid per lane using the *Telo TAGGG* Telomere Length Assay kit (Roche, Mannheim, Germany), as previously described [21]. TRFs were measured in duplicate for 22 individuals, in triplicate for five, in quadruplicate for 14, in quintuplicate for one, and only once for five controls. Images were analysed with IMAGEQUANT TL v2003 (Amersham Biosciences, Piscataway, NJ, USA) according to *Telo TAGGG* kit's instructions.

Telomeric single-stranded overhang length measurement

Telomeric overhangs were assessed by two different methods: the native or non-denaturing method – as in the above-described Southern blot for overall telomere length, but in which DNA is not denatured and only single strands hybridize to the probe, as previously reported

[22] – and the recently described telomere-oligonucleotide ligation assay (T-OLA) [18,21,23].

For the non-denaturing method, analysis of overhangs was performed in triplicate for patients C and E, and in duplicate for all the other patients as well as for patient D's kindred; for healthy volunteers, measurement was performed in quadruplicate for three and just once for the others. Images were analysed using IMAGEQUANT TL after background subtraction. The same three controls were present in every gel and the average of their net volume calculated as 1.

With the T-OLA assay, overhang length was measured in duplicate for twelve healthy controls, for patient C and one sister, patient F and patient D and his family. Images were analysed using IMAGEQUANT TL. Intensity of the ladder adds up to 100%; intensity of each band – corresponding to concatenated oligonucleotides – was calculated as its relative frequency in comparison with the ladder, as previously described [23].

Statistical analysis

The length of telomeric overhangs was quantitated using IMAGEQUANT TL v2003 (Amersham Biosciences) and compared between controls and patients by employing the Mann-Whitney nonparametric test using GRAPHPAD PRISM 4 (GraphPad Software, San Diego, CA, USA), considering a *P*-value < 0.05 as statistically significant. Fifth and ninety-fifth percentiles for telomeric overhang length of healthy controls were calculated.

Results

Telomeric single-stranded overhang lengths are maintained with ageing

Among healthy controls, overall telomeric length of leucocytes shortened as a function of age from umbilical cord blood to 76-year-old healthy volunteers following a third-order polynomial (Fig. 2a,b). However, single-stranded overhang length was maintained with ageing as determined by both non-denaturing (Fig. 2c) and the T-OLA (Fig. 1d) methods.

Telomeric single-stranded overhangs are shorter in telomerase-mutant patients with aplastic anaemia

As telomeric overhangs were maintained in physiological telomere shortening in which telomerase activity is maintained, we explored whether telomeric overhang structure was abnormal in the pathological state of defective telomerase function and telomere shortening, previously described in human AA. We analysed the length of 3'-G rich overhangs of 10 patients in whom we previously had identified telomerase mutations known to modulate telomerase activity (Table 1) [16,17,24,25] (and from whom enough DNA was available for experiments) and found significant overhang shortening in comparison with age-matched controls (*P* < 0.05; Mann-Whitney test; Fig. 3a): seven telomerase complex-mutant patients with AA had overhang lengths below the 5% percentile for healthy volunteers (Fig. 3a).

In family members of patient G, who have normal overall telomere length for his age, short telomeric overhangs were detected only in the patient's mother, who does not carry the TERT codon deletion (Fig. 3a, left panel). However, there is a strong family history of AA

on the mother's side of the family, suggesting that genetic factors other than the *TERT* variant found in the proband also are associated with overhang erosion. In patient E's kindred, short overhangs were restricted to the patient's leucocytes only (Fig. 3a, middle panel).

To clarify whether short overhangs were caused by the marrow failure itself and not to abnormal telomerase, we selected five patients who had responded robustly to immunosuppression, had normal genotype for *TERT*, *TERC*, *TERF1* and *TERF2*, and normal length of overall telomeres (Table 1) for measurement of overhang lengths in the peripheral blood samples (collected at diagnosis and not at the time of haematopoietic recovery). Telomeric overhangs were similar in length between this group of AA patients and healthy individuals, and all values fell within fifth and ninety-fifth percentiles of healthy control values (data not shown).

Discussion

In this study, we showed that telomeric overhang lengths of peripheral blood leucocytes are maintained with ageing despite overall telomere length attrition. In human cells, telomeric single-stranded overhangs vary in length from 10 to 100 hexameric repeats and are produced by both the addition of telomeric repeats to the 3' end of telomeric DNA by telomerase and post-replicative processing of telomeric ends [26,27]. Our results indicate that telomeric overhangs do not shorten with ageing, and they are in agreement with recent *in vitro* observations that in normal human BJ foreskin and IMR90 foetal lung fibroblasts, overhang lengths are constant at cell senescence [28] and that human fibroblasts preserve telomeric overhang lengths in culture despite overall telomere shortening [29]. These results taken together indicate that telomeric overhang attrition does not appear to correlate with overall telomere shortening, to play a role in the physiological process of senescence of human haematopoietic cells, nor is it a consequence of ageing.

However, in pathological states associated with reduced telomerase activity because of loss-of-function telomerase mutations, we found that telomeric overhangs were significantly eroded. These findings are consistent with very recent results in a family with Hoyeraal-Hreidarsson syndrome (HHS), a very severe variant of DKC associated with neurological defects [30]. In this pedigree, no known telomerase complex or shelterin mutation was identified and telomerase enzymatic activity did not appear reduced in patient's primary cells. Although telomeres were normal in length in patient's primary fibroblasts, they activated DNA-damage response and showed severely eroded overhangs, similar to the findings in patient G's peripheral blood leucocytes.

The mechanism of telomeric overhang generation in human cells still is poorly understood. Leading and lagging strand overhangs are differentially synthesized and telomerase participates in the extension of leading-strand overhangs, but not in lagging daughter telomeres [31]. Telomerase also is thought to contribute to telomeric overhang formation by addition of telomeric repeats to the 3' overhang [26]. Our results suggest that telomerase deficiency may contribute to overhang shortening in AA patients but is not sufficient, as healthy mutation carriers have normal overhang lengths; either marrow environmental or

genetic factors also are necessary for deficient overhangs. That overhang erosion is not a consequence of the disease pathogenesis (bone marrow stress caused by the immune attack to haematopoietic precursors and apoptosis) is indicated by normal overhang lengths in patients with AA with normal overall telomere length normal genotype for telomerase genes.

More important, along with recent findings in HHS [30], our results indicate that short telomeric overhang contribute to cellular senescence in bone marrow failure syndromes. Eroded telomeric overhangs may alter telomere structure by disrupting the T-loop, thus activating DNA-damage responses, as indicated in HHS [30]. Patients with telomerase mutations and short telomeres are more likely to eventually develop myelodysplasia and acute myeloid leukaemia [3,32] and there is a significant association between trisomy 8 and inversion 16 and telomerase mutations in acute myeloid leukaemia patients [25]. In telomerase deficient mouse models, telomere dysfunction appears to promote chromosomal translocations and epithelial cancers via bridge-fusion-bridge processes [33]. Disturbance in the T-loop formation is likely facilitate activation of DNA repair machinery, end-to-end fusions, bridge-fusion-breakage, aneuploidy, thus contributing to chromosomal instability and malignant transformation [3,25]. In conclusion, short telomeric overhangs may contribute to pathological states associated with abnormal telomere maintenance but not to overall telomere shortening observed with physiological ageing.

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Figure 1. Schematic representation of telomere structure. The telomeric DNA 3'-G overhang is single stranded (red) and essential for t-loop formation by intertwining the duplex telomere (reprinted from Calado RT and Young NS. Blood 111: 4446–55).

Figure 2.

Overall telomere and telomeric overhang lengths with ageing. (a) Overall telomere length of peripheral blood leucocytes of healthy subjects measured by Southern blot, demonstrating that telomere length shortens with ageing from umbilical cord blood (CB) until 76 years of age (ages in years on top of lanes), as plotted as a function of age in (b) (MW, molecular weight marker). (c) Telomeric single-stranded overhang in peripheral blood leucocytes of healthy individuals measured by conventional nondenaturing method. Overhang size does not change as a function of age. Blue bars in the graphic below represent the relative net volumes obtained in the analysis of non-denaturing gel and correspond to overhang signal normalized for background. Orange shade corresponds to 95% confidence interval for relative net volume in healthy individuals. (d) Telomeric single-stranded overhang in peripheral blood leucocytes of healthy individuals measured by T-OLA assay, also indicating

that overhang lengths do not change with ageing. GAPD corresponds to DNA loading control and graphic below indicates the frequency distribution of telomeric repeats in each age group; the x-axis corresponds to the number of oligonucleotides ligated. The number of ligated oligonucleotides directly correlates with the length of telomeric overhangs.

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Figure 3.

Telomeric overhang length in patients with aplastic anaemia. (a) Telomeric overhang length in patients with acquired aplastic anaemia carrying telomerase mutations and relatives measured by conventional non-denaturing method. Letters correspond to patient identification in Table 1. Middle panel, patient E's kindred. Blue bars in the graphic below represent the relative net volumes obtained in the analysis of non-denaturing gel above and correspond to overhang signal normalized for background. Orange shade corresponds to 95% confidence interval for relative net volume in healthy individuals (Fig. 2). Seven of ten mutant patients studied had telomeric overhangs below the 95% confidence interval for healthy controls. (b) Telomeric overhang lengths of patients and relatives measured by T-OLA. The gel on the left shows the analysis from patient G in the first lane, a healthy control DNA digested with *ExoI* (a single-stranded 3' specific exonuclease), and healthy control DNA without digestion (NE). Right gel illustrates overhang length in U937 cells, a 31-year-old healthy control, patients G, J, and E, and patients G and E's kindred, showing shorter telomeric overhangs in patient G. GAPD corresponds to DNA loading control. Graphic below shows the frequency distribution of telomeric repeats for healthy controls (white bars; mean $\pm$ standard error) and patient G (blue bars), indicating that patient G has a higher percentage of overhangs with only one repeat and lacks longer overhangs with multiple (>6) repeats.

Table 1
Clinical and laboratory profiles of patients with acquired aplastic anaemia carrying telomerase gene mutations

Patient	Mutation	Age (years)	Sex	Race or ethnicity	Diagnosis	Hb (g dL ⁻¹)	ANC	Platelet	BM cellularity (%)	Telomere length* (kb)	Family history	Follow up
Patients with telomerase mutations												
A	<i>TERC</i> A117C	39	M	Caucasian	sAA	8.1	2.8	4	10–20 [†]	4.5	Father with aplastic anaemia; brother with diabetes	Partial response to androgens; died of infection
B	<i>TERC</i> del28–34	29	M	Caucasian	sAA	10.1	1.5	44	10–20	4.2	Father with pulmonary fibrosis, liver cirrhosis, marrow failure; uncle with myelodysplasia; brother with hypoplastic anaemia	Failed to courses of IST; responded to androgens
C	<i>TERC</i> del341–360	57	M	Caucasian	sAA	7.9	2.8	32	5	3.6	Liver cirrhosis and oesophageal cancer; son with liver disease and hypoplastic bone marrow	Oesophageal cancer; died of cancer progression
D	<i>TERC</i> 109–123	38	F	Caucasian	mAA	9.7	0.9	68	<5	4.0	Family history of liver cirrhosis	Not treated
E	<i>TERT</i> A202T	41	M	Hispanic	mAA	8.3	1.3	6	20	4.8	-	Transient response to IST; died of transplant complications
F	<i>TERT</i> G260D	73	M	Caucasian	sAA	9.4	1.1	20	20	5.0	-	No response to IST; died at hospice
G	<i>TERT</i> del441E	24	M	Caucasian	mAA	11.2	0.7	31	10	8.1	Aunt with aplastic anaemia	Partial response after second course of IST
H	<i>TERT</i> K570N	26	M	Caucasian	sAA	9.8	1.2	20	15	3.1	Father with AML; aunt with aplastic anaemia	No response to either IST or androgens
I	<i>TERT</i> R889X	38	F	Hispanic	sAA	8.2	3.3	87	15	5.1	Father and sister with pulmonary fibrosis	Partial response to androgens
J	<i>TERT</i> V1090M	64	F	Hispanic	sAA	10.1	0.3	3	<10	6.0	-	No response to IST
Responders to immunosuppression												
K	-	18	M	Caucasian	AA	7.1	1.0	10	50	7.2	Mother with pregnancy-related thrombocytopenia	Complete response to IST
L	-	26	M	Caucasian	AA	8.2	0.2	14	<5	6.7	-	Complete response to IST
M	-	30	M	Hispanic	AA	7.9	1.0	12	10	6.6	-	Complete response to IST

Patient	Mutation	Age (years)	Sex	Race or ethnicity	Diagnosis	Hb (g dL ⁻¹)	ANC	Platelet	BM cellularity (%)	Telomere length* (kb)	Family history	Follow up
N	-	36	F	Caucasian	sAA	9.9	1.1	34	10	6.6	-	Complete response to IST
0	-	79	M	Caucasian	sAA	7.8	0.1	5	<10	5.9	-	Partial response to IST

sAA, severe aplastic anaemia; mAA, moderate aplastic anaemia; IST, immunosuppressive therapy; AML, acute myeloid leukaemia.

* Telomere lengths for peripheral blood leucocytes of patients carrying TERC or TERT mutations (A–F) were already published elsewhere [16,17,24] and determined by flow-FISH (interpretation corresponds to length in comparison to age-matched healthy controls); for responder patients (G–K), telomere length was assessed using Southern blot and telomere restriction fragment (TRF) length measurement (see Materials and methods).

† Patient A had normal cytogenetic study at presentation but developed aneuploidy [+(1)(q10)] 15 years after diagnosis.