Abnormal telomere shortening of peripheral blood mononuclear cells and granulocytes in patients with chronic idiopathic neutropenia

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ABSTRACT

Background
Chronic idiopathic neutropenia is characterized by immune-mediated suppression of neutrophil production. Because patients with immune-mediated bone marrow failure syndromes display age-inappropriate telomere shortening in leukocytes, we investigated telomere lengths in peripheral blood mononuclear cells and granulocytes of patients with chronic idiopathic neutropenia.

Design and Methods
We studied 37 patients with chronic idiopathic neutropenia and 68 age- and sex-matched healthy controls. Relative telomere length and telomerase reverse transcriptase expression were assessed by a quantitative real time polymerase chain reaction. Telomerase activity was determined by a polymerase chain reaction-based immunoassay.

Results
The mean relative telomere values of peripheral blood mononuclear cells and granulocytes were significantly lower in patients compared to controls, and significantly lower than expected on the basis of the age-adjusted healthy control distribution. The difference in the relative telomere lengths between patients and controls in both peripheral blood mononuclear cells and granulocytes was prominent in those under the age of 50 years. Contrary to the peripheral blood mononuclear cells, in which an inverse correlation was observed between relative telomere values and age, no significant correlation was noted between granulocyte telomere values and patient age. A significant correlation was observed between individual relative telomere values and absolute neutrophil counts. There was no difference in expression of telomerase reverse transcriptase in peripheral blood mononuclear cells between patients and controls but telomerase activity was identified at a significantly higher frequency in controls than in patients. No correlation was found between telomerase activity or telomerase reverse transcriptase expression and relative telomere lengths of peripheral blood mononuclear cells.

Conclusions
Patients with chronic idiopathic neutropenia display age-inappropriate telomere shortening of peripheral blood cells and low telomerase activity in peripheral blood mononuclear cells. A compensatory increased proliferation of bone marrow hematopoietic progenitor cells in association with lymphocyte replicative exhaustion probably account for these abnormalities.

Key words: chronic idiopathic neutropenia, telomere shortening, peripheral blood mononuclear cells, granulocytes, age.

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Introduction

Chronic idiopathic neutropenia (CIN) in adults is an acquired disorder of granulopoiesis characterized by prolonged 'unexplained' reduction in the number of circulating neutrophils, and a usually benign and uncomplicated disease course.[1,2] Defective neutrophil production in CIN has been associated with increased Fas-mediated apoptosis of the granulocytic progenitor cells within an inhibitory bone marrow (BM) microenvironment consisting of pro-inflammatory cytokines, such as tumor necrosis factor-α, interferon-γ, interleukin-1β, interleukin-6, and pro-apoptotic mediators, such as Fas-ligand, CD40-ligand and transforming growth factor-β1 among others.[3-5] As far as the cellular origin of these inhibitory molecules is concerned, it has been shown that activated monocytes present in CIN BM play a major role through upregulation of toll-like receptor 4 and activation of the downstream signal transduction pathway leading to pro-inflammatory cytokine production.[6] However, the most important cellular constituent of the inflammatory BM microenvironment in CIN is the T-cell component. It has been shown that activated T-cell subsets with a skewed oligoclonal/monoclonal expansion pattern persist in CIN BM and display myelosuppressive properties *in vitro*.[7,8] Patient peripheral blood (PB) T lymphocytes also show an activated phenotype with increased rate of proliferation and apoptosis indicative of antigen-driven immune responses.[8] Overall, it has been proposed that CIN shares pathophysiological features with common immune-mediated BM failure syndromes, such as acquired aplastic anemia (AA), large-granular lymphocyte proliferative disease and myelodysplastic syndromes (MDS).[9,10] The suppression and dysplasia of the BM erythroid and megakaryocytic lineages in CIN, albeit to a mild degree, further corroborate the assumption that CIN represents a mild form of the spectrum of immune-mediated BM failure syndromes.[11,12]

Abnormal telomere shortening of PB leukocytes has been described in patients with acquired immune-mediated BM failure syndromes including AA, paroxysmal nocturnal hemoglobinuria (PNH) and MDS; this abnormality is thought to play a prominent role in the premature cellular senescence, the accelerated apoptosis, genomic instability and abnormal replication associated with these disease states.[13-17] The rapid turnover of hematopoietic progenitor cells in an attempt to compensate for the failing BM has been recognized as the main underlying mechanism for the short telomeres in these disease entities.[16]

The telomere length of PB cells in patients with CIN has not been extensively studied. We examined the telomere length of PB mononuclear cells (PBMCs) and granulocytes in a cohort of well characterized patients with CIN using a quantitative polymerase chain reaction (PCR).[18] Through the analysis of telomere length in PB hematopoietic cells, we aimed to determine whether premature telomere loss was an additional feature that CIN shares with other immune-mediated BM failure syndromes.

Design and Methods

Patients and controls

We studied 37 adults with CIN, 5 males and 32 females aged 25-70 years (median age 49 years), who satisfied the previously defined diagnostic criteria for the disease.[3] In particular, the patients had neutrophil counts less than 1800×10^6/L (mean $1209\pm397\times10^6$/L, range $300\text{-}1760\times10^6$/L) for a period of 10–240 months (median duration 84 months), had no clinical, serological or ultrasonic evidence of any underlying disease known to be associated with neutropenia, no history of exposure to irradiation, use of chemical compounds or intake of drugs to which neutropenia might be ascribed, normal BM karyotype, and negative serum leukoagglutination and immunofluorescence tests for antineutrophil antibodies. Cyclic and familial neutropenias were excluded by performing serial neutrophil enumerations in patients and their family members. Detailed patients' characteristics are shown in Table 1. Patients with neutrophil counts over 1500×10^6/L might be considered as borderline neutropenic cases. As controls, 68 hematologically healthy subjects, age- and sex-matched with the patients, were studied; the control group consisted of 9 male and 59 female healthy subjects aged 30-73 years (median age 46 years). The mean neutrophil count in the control population was $4213\pm1156\times10^9$/L (median 4050×10^9/L, range $2100\text{-}4900\times10^9$/L). The study was approved by the ethics committee of the University Hospital of Heraklion and informed consent according to the Declaration of Helsinki was obtained from all study subjects.

Cell separation

PB samples were drawn from all subjects in tubes containing ethylenediaminetetraacetic acid (EDTA). The PBMCs and granulocytes were isolated by dextran sedimentation and gradient centrifugation of the leukocyte-rich plasma on Histopaque-1077 (Sigma, Saint Louis, MO, USA) followed by lysis of contaminating red blood cells by ammonium chloride.[19]

Real time quantitative PCR for telomere length measurement

DNA was extracted from PBMCs and neutrophils using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany). β-globin was used as control single-copy gene.[18] SYBR GreenER qPCR Supermix (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for the reactions. The forward and reverse primer sequences for telomere PCR amplification were: 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3′ and 5′-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′′, respectively. For β-globin, the forward and reverse primer sequences were: 5′-GCTTCTGACACAACTGTGTTCACTAGC-3′ and 5′-CACCAACTTCATCCACGTTCACC-3′, respectively. For PCR amplifications, reactions were incubated for 10 min at 95°C and then amplified over 40 cycles of 15 s at 95°C and 60 s at 56°C (for telomeres) or 58°C (for β-globin). Reactions were run in a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). All samples were processed in triplicate. A standard curve was generated using serially diluted genomic DNA. Telomere length was reflected by the relative telomere/single copy gene ratio (T/S) values: $T/S=2^{-\Delta Ct}$ ($\Delta Ct = Ct^{telomere} - Ct^{\beta\text{-}globin}$).

Telomerase activity

Telomerase activity in extracts of PBMCs and granulocytes of CIN patients and healthy controls was determined using the highly sensitive TeloTAGGG Telomerase PCR ELISA kit (Roche Diagnostics Scandinavia AB, Stockholm, Sweden), a specific photometric enzyme immunoassay based on the Telomeric Repeat Amplification Protocol (TRAP).[20,21] Reactions were performed with 50 μg of total cell protein for each sample according to the manufacturer's instructions. Immortalized telomerase-expressing human kidney cells were used as positive control. Cellular extracts after heat treatment (10 min at 85°C) were used as negative control.

Human telomerase reverse transcriptase (hTERT) *gene expression by real time quantitative PCR*

For the evaluation of hTERT expression, total cellular RNA was isolated from PBMCs and granulocytes using the RNeasy Mini Kit (Qiagen). From each PBMC and granulocyte sample, 1 μg of RNA was transcribed into cDNA by means of the SuperScript II First-Strand cDNA synthesis kit (Invitrogen Life Technologies). Then 100ng of cDNA were amplified by real time PCR using SYBR GreenER qPCR Supermix (Invitrogen Life Technologies) and 800nM of each primer. Reactions were run in a Rotor-Gene 6000 using a two-step cycling program that consisted of: 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 15 s. A melting curve (62-95°C) was generated at the end of each run to verify specificity of the reaction. The forward and reverse primer sequences for hTERT PCR amplification were: 5'-TGTACTTTGT-CAAGGTGGATGTG-3' and 5'-TGGAGGTCTGTCAAGGTA-GAG-3', respectively. GAPDH was used as control gene and the forward and reverse primer sequences for PCR amplification were: 5'-GCCCAATACGACCAAATCC-3' and 5'-AGCCA-CATCGCTCAGACA-3', respectively. The expression of hTERT was calculated according to the $2^{-\Delta CT}$ method ($\Delta Ct = Ct^{hTERT}-Ct^{GAPDH}$). The fold change (FC) of hTERT expression in the group of patients compared to controls was calculated with the ΔΔCt method where $FC=2^{-\Delta\Delta Ct}$ and $\Delta\Delta Ct = [(Ct^{hTERT}-Ct^{GAPDH})CIN - (Ct^{hTERT}-Ct^{GAPDH})normal]$.[22,23]

Statistical analysis

Data were analyzed using GraphPad Prism statistical software (GraphPad Software, San Diego, CA, USA). The χ^2 test was used to determine the homogeneity of the populations regarding the age (P=0.493) and sex (P=0.968) distribution, as well as to determine differences in the proportion of patients and controls displaying telomerase activity or hTERT expression in PBMCs. The Mann-Whitney U test was used to compare the mean relative telomere length or hTERT values between PBMCs and granulocytes or between patients and controls. The two-way analysis of variance test was used to compare the mean relative telomere values in different age groups between patients and controls. Finally, Spearman's correlation test was used to define the relationships between telomere lengths and a variety of clinical/laboratory parameters. Values are expressed as means ±1 standard deviation.

Results

Telomere length analysis of PBMCs in CIN patients and healthy controls

The mean relative telomere length (T/S ratio) of PBMCs was significantly lower in CIN patients (4405.86±1952.14) compared to healthy controls (6492.12±4125.34; P=0.0183). As anticipated, a statistically significant inverse correlation was observed between the relative telomere length of PBMCs and age in both healthy subjects (r = -0.408; P=0.0006) and CIN patients (r=-0.456; P=0.0045) (Figure 1A and B). To further characterize patients' PBMC relative telomere length as appropriate or inappropriate for a given age, we defined the observed/predicted relative telomere length ratio (O/P ratio) for each patient according to the equation derived from the linear regression analysis of the correlation between the PBMC relative telomere values and age (years) of the controls (y = -158.18x + 13813).[24] We found that the mean O/P telomere length ratio of the patients (0.73±0.32) was out of the 95% confidence limits (CI) of the healthy controls (mean O/P telomere length ratio 1.01 ±0.56, 95% CI 0.87 and 1.14) suggesting inappropriate telomere loss according to age in CIN patients (Figure 1C). Overall, the mean telomere length of patient PBMCs was significantly lower than that expected on the basis of the age-adjusted healthy control distribution (6203.26±1871.90; P=0.0004). Analysis of the mean PBMC relative telomere length per decade of years in patients and controls showed a highly significant difference (F=4.374; P=0.0391) further corroborating the premature telomere loss of PBMCs in CIN patients (Figure 1D). By analyzing separately the mean telomere length in the younger (≤ 50 years) and older (> 50 years) age groups, we found that

Table 1. Clinical and laboratory data of the patients studied.

UPN	Age (years)	Sex	Duration (months)	Hb (g/dL)	WBC (×10⁶/L)	Neutro (×10⁶/L)	Lympho (×10⁶/L)	Mono (×10⁶/L)	Plts (×10⁹/L)
1	70	M	80	14.3	2500	800	1300	300	163
2	68	F	12	13.6	3000	1400	1300	200	263
3	65	F	225	12.6	3000	1700	1100	100	222
4	65	F	94	13.4	3100	700	1900	400	175
5	64	F	91	14.1	2914	664	1746	404	169
6	65	F	229	12.6	2800	1500	1100	100	173
7	60	F	80	12.7	4200	1600	2200	300	225
8	59	F	111	12.4	2000	500	1300	100	238
9	58	F	36	13.4	2800	1500	1100	100	182
10	53	F	36	13.9	3700	1628	1739	233	218
11	52	F	73	12.4	3100	1300	1300	400	290
12	51	F	228	10.9	3700	1300	1900	400	243
13	58	F	180	14.0	3300	1600	1400	200	196
14	56	F	112	12.5	2100	600	1300	100	188
15	52	F	75	12.4	3000	1200	1300	400	197
16	51	F	150	14.0	3000	1200	1100	600	200
17	49	F	85	11.0	2400	1500	700	100	184
18	50	F	48	12.4	4000	1760	1760	380	171
19	49	F	240	12.0	2400	1030	960	310	190
20	41	F	84	13.3	3300	1100	1600	500	264
21	41	F	84	11.3	3000	1100	1300	500	219
22	45	F	202	11.4	3400	1700	1381	219	189
23	42	F	204	13.4	3000	1100	1500	300	263
24	45	M	50	15.0	4500	1300	2600	500	163
25	45	M	10	14.3	5900	1593	3422	785	222
26	45	F	200	12.4	2900	1100	1400	300	245
27	42	F	79	14.5	2000	500	1100	300	269
28	41	F	84	11.3	3000	1100	1300	500	173
29	37	M	120	14.5	2922	300	1924	598	225
30	40	F	36	13.9	2900	1500	1100	200	238
31	37	F	48	12.0	2000	1100	700	100	182
32	31	F	50	14.0	2500	900	1300	200	218
33	31	F	24	12.4	2560	800	1460	200	190
34	31	F	72	12.7	3300	1200	1600	400	243
35	36	F	156	12.9	3200	1400	1400	300	196
36	30	M	120	14.4	3500	1750	1470	180	188
37	25	F	60	13.9	4200	1600	2100	400	257

UPN: unique patient number; Hb: hemoglobin; WBC: white blood cells; Neutro: neutrophils; Lympho: lymphocytes; Mono: monocytes; Plts: platelets.

patients showed significantly shorter telomeres compared to controls mainly in the younger group (5063.27±1767.77, n=21 *vs.* 7590.46±4411.92, n=45; *P*=0.0149) (Figure 1D). Patient telomere lengths (3543.02±1892.14, n=16) were also decreased compared to controls (4343.19±2367.16, n=23) in the older age group, but this was not statistically significant (*P*=0.4493) (Figure 1D).

Telomere length of granulocytes in CIN patients and healthy controls

A statistically significant difference in the mean relative telomere length of granulocytes was seen between CIN patients and controls: this was lower in CIN patients (3037.98±1278.84) than in controls (4874.74±4049.99; *P*=0.0352). Individual relative telomere values in patients were significantly lower than expected on the basis of the age-adjusted normal distribution (4649.13±1461.38; *P*<0.0001). Interestingly, a statistically significant inverse correlation was obtained, as expected, between individual relative telomere values of granulocytes and age in the group of controls (y=-123.49x + 10590; r = - 0.324; *P*=0.007) but not in the group of patients (y=-8.4585x + 3444.9; r = -0.078; *P*=0.645) (Figure 2A and B). The mean O/P telomere length ratio of patient granulocytes (0.73±0.49) was out of the 95% CI limits of the healthy control distribution (mean O/P telomere length ratio 1.01±0.56; 95% CI 0.83 and 1.22) suggesting inappropriate telomere loss according to age in patient granulocytes (Figure 2C). Analysis of the mean relative granulocyte telomere length per decade of years in patients and controls showed no statistically significant difference between the two distributions (F = 3.554; *P*=0.062). However, a subset analysis showed statistically significantly lower mean relative telomere length in patients compared to controls in the younger age groups, *i.e.* in the group under the age of 50 years (2979.92±773.09, n=21 *vs.* 5714.09±4303.43; n=45; *P*=0.0016) but not in the groups over the age of 50 years (3114.1 9±1765.56; n=16 *vs.* 3232.54±2941.66; n=23; *P*=0.6376) (Figure 2D). All the above data indicate a telomere length deficit in the granulocytes of CIN patients compared to age-matched healthy individuals mainly attributed to a significant telomere loss in the younger age groups.

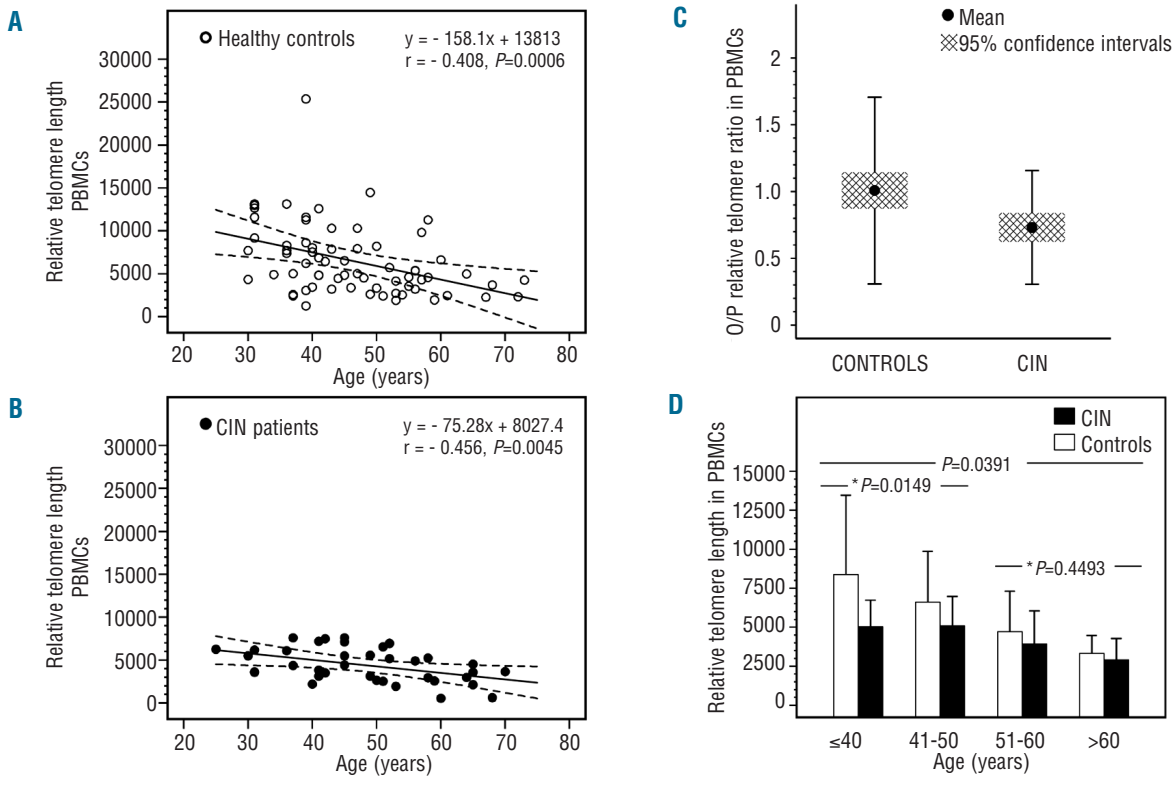

Figure 1. Relative telomere length values of PBMCs in CIN patients and age/sex-matched healthy controls. (A-B) Correlation (regression line ±95% confidence limits) between the relative telomere length values of PBMCs and age in the healthy controls and CIN patients, respectively. Analysis performed by Spearman's correlation test. The equation, coefficient of correlation (r) and degree of significance (*P*) are indicated. (C) Observed/predicted relative telomere length ratio (O/P ratio) values for patients and controls. Individual predicted values in patient and control groups were estimated from the equation derived from the linear regression analysis of the correlation between the relative telomere length values and age of the controls shown in (A). The dots ± error bars represent the mean O/P ratio±1SD for each group and the floating bars represent the 95% confidence intervals for each distribution. The mean O/P ratio the in the patient group is from the 95% confidence intervals of the normal group suggesting inappropriate telomere loss by age in CIN. The bars (D) show the mean (+1SD) relative telomere length per decade of years in CIN patients and healthy controls. The comparison between patient and control telomere length distributions has been performed by two-way analysis of variance test which compares multiple mean values; the statistically significant difference (*P*=0.0391) is shown. A subset analysis of telomere length between patients and controls in the younger (≤ 50 years) and older (>50 years) age groups was performed by means of the Man-Whitney U test. A statistically significant difference was demonstrated in the younger but not the older groups and the respective *P* values are shown with asterisks. PBMCs: peripheral blood mononuclear cells; CIN: chronic idiopathic neutropenia; SD: standard deviation.

Correlations of telomere length with blood counts and disease duration

A significant positive correlation was observed between the mean relative telomere length of granulocytes and PBMCs in the group of healthy controls (r = 0.3243; *P*=0.007) but not in patients. In accordance with previously reported data,[25,26] in the group of healthy controls the mean relative telomere length was significantly lower in the granulocytes (4874.74±4049.99) compared to PBMCs (6492.12±4125.34; *P*=0.0032) in both the younger (under the age of 50 years) (5714.09±4303.43 *vs.* 7590.46±4411.92, respectively; *P*=0.0144) and the older age groups (3232.54±2941.66 *vs.* 4343.19±2367.16, respectively; *P*=0.023) (Figure 3A). In the group of CIN patients, the mean telomere length was also significantly lower in the granulocytes (3037.98±1278.84) compared to PBMCs (4405.86±1952.14; *P*=0.003) mainly due to the lower telomere length of granulocytes compared to PBMCs in the younger group of patients (2979.92±773.09 *vs.* 5063.27±1767.77, respectively; *P*=0.0001). In patients over 50 years of age, the mean telomere length of granulocytes was also lower compared to PBMCs but this was not statistically significant (3114.19±1765.56 *vs.* 3543.02±1892.14, respectively; *P*=0.5848) (Figure 3B). The previously described presence of activated T lymphocytes in patients' PBMC fraction, known to display shorter telomere lengths compared to naïve T lymphocytes, in association with our recent data showing accelerated telomere loss by age in PB T-cell subsets of CIN patients, may explain this deviation in the older patient group.[27,28] Finally, no significant correlation was observed in the group of patients between the relative telomere length of PBMCs or granulocytes and disease duration, white blood cell counts, lymphocytes and monocytes (*data not shown*). However, a significant correlation was observed between granulocyte relative telomere values and absolute neutrophil counts (r = 0.3798; *P*=0.0204) suggesting that a decline in telomere-length correlates with the severity of neutropenia in CIN (Figure 4).

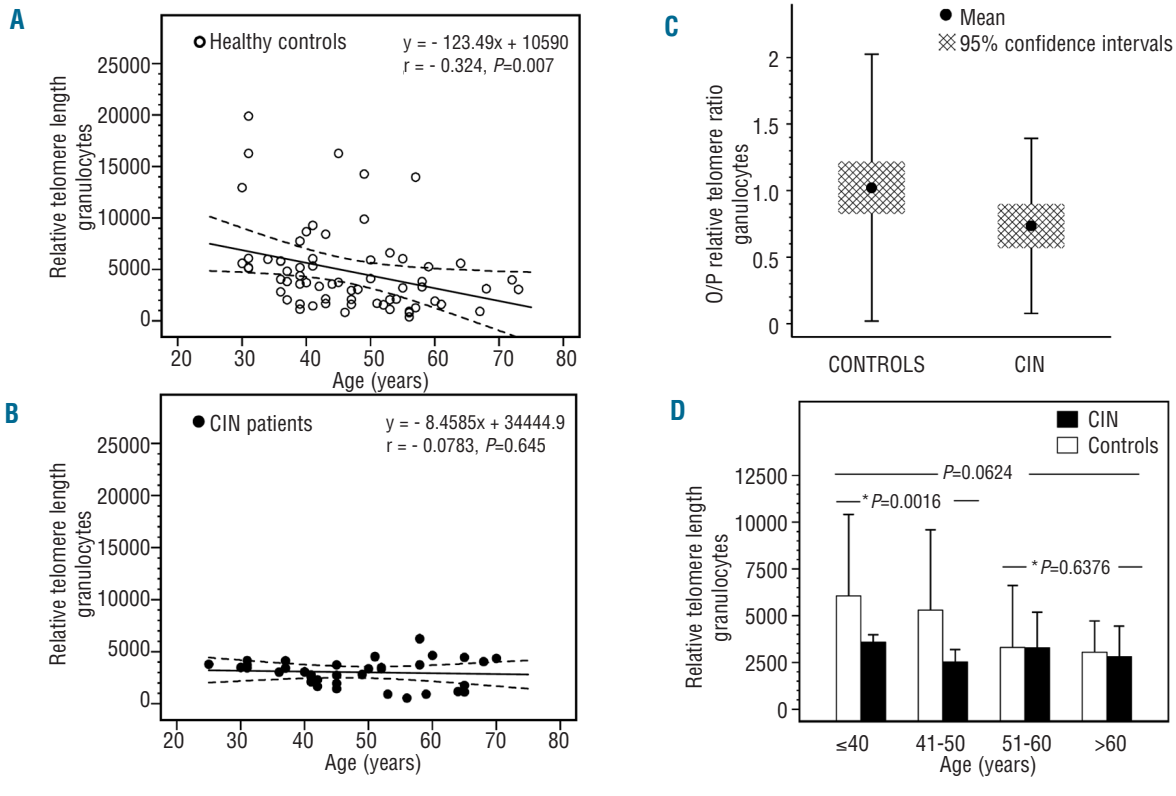

Figure 2. Relative telomere length values of granulocytes in CIN patients and age- and sex-matched healthy controls. (A-B) Correlation (regression line ± 95% confidence limits) between the relative telomere length values of granulocytes and age in the healthy controls and CIN patients, respectively. Analysis has been performed by Spearman's correlation test. The equation, coefficient of correlation (r) and degree of significance (*P*) are indicated. (C) Observed/predicted relative telomere length ratio (O/P ratio) values for patients and controls. Individual predicted values in patient and control groups were estimated from the equation derived from the linear regression analysis of the correlation between the relative telomere length values and age of the controls shown in (A). The dots ± error bars represent the mean O/P ratio±1SD for each group and the floating bars represent the 95% confidence intervals for each distribution. The mean O/P ratio in the patient group is from the 95% confidence intervals of the normal distribution suggesting inappropriate telomere loss by age in CIN. The bars in (D) show the mean (+ 1SD) relative telomere length per decade of years in CIN patients and healthy controls. The comparison between patient and control telomere length distributions was performed by the two-way analysis of variance test (which compares multiple mean values) and did not show a statistically significant difference (*P*=0.0624). A subset analysis of telomere length between patients and controls in the younger (≤ 50 years) and older (> 50 years) age groups was performed by means of the Mann-Whitney U test. A statistically significant difference was demonstrated in the younger but not the older group and the respective *P* values are shown with asterisks. CIN: chronic idiopathic neutropenia; SD: standard deviation.

Telomerase activity and hTERT gene expression in PBMCs and granulocytes of CIN patients and healthy individuals

To investigate whether the lower telomere length of PB cells in CIN patients might be due to alterations in telomerase, we evaluated the telomerase activity and hTERT expression in PBMCs and granulocytes from CIN patients and healthy individuals. Consistent with previously reported data, no telomerase activity or hTERT mRNA expression was identified in the granulocytes of any of the subjects studied.[26,27,29,30] As far as PBMCs are concerned, 7 CIN patients (18.92%) and 34 healthy controls (50%) showed detectable telomerase activity (P=0.0018). Given that the monocytes do not display telomerase activity,[27,31] these data suggest that PB lymphocytes of CIN patients display lower telomerase activity compared to healthy individuals. Expression of the hTERT gene in PBMCs was identified, albeit at low levels, in 23 CIN patients (62.16%) and 42 healthy controls (61.76%) suggesting no statistically significant difference between the two groups. Among the hTERT expressing subjects, there was no significant difference in mean relative hTERT expression between patients and controls (P=0.11); observed hTERT FC expression in patients compared to controls was 1.299. In both patient and control groups, all subjects with detectable telomerase activity also showed hTERT expression. No correlation was found between telomerase activity or hTERT expression and relative PBMC telomere length in either patients or healthy subjects suggesting that factors other than TERT predominate in telomere length modulation in these cell populations.

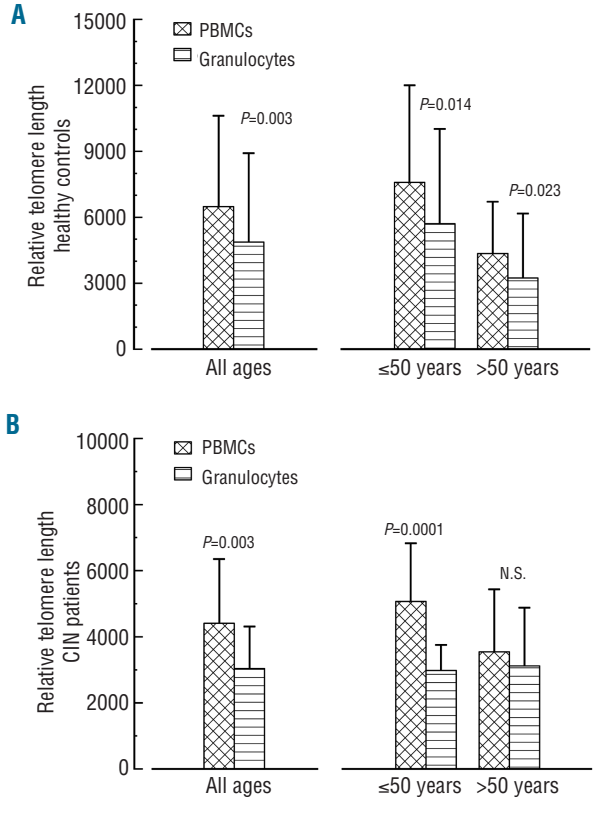

Figure 3. Relative telomere length values of PBMCs and granulocytes in CIN patients and age- and sex-matched healthy individuals. The bars represent the mean (+ 1SD) relative telomere length in PBMCs and granulocytes of healthy individuals (A) and CIN patients (B). The left bars in each graph correspond to the entire group of healthy subjects or CIN patients whereas the bars on the right show the mean relative telomere length in the younger (≤ 50 years) and older (> 50 years) groups of healthy individuals and CIN patients. Comparison between relative telomere lengths of PBMCs and granulocytes was performed by the Mann-Whitney U test and the statistically significant P values are shown. PBMCs: peripheral blood mononuclear cells; CIN: chronic idiopathic neutropenia; SD: standard deviation; N.S.: non-statistically significant differences.

Discussion

Patients with BM disorders mediated by activated lymphocytes and/or pro-inflammatory cytokines and pro-apoptotic molecules, such as acquired AA, PNH and MDS, have been reported to show abnormal telomere shortening of PB hematopoietic cells.[13-15,32,33] This abnormality, reflecting the accelerated telomeric erosion of hematopoietic stem cells from which leukocytes are derived, has been primarily attributed to the rapid turnover of the hematopoietic stem/progenitor cells in an attempt to compensate insufficient or ineffective cell production. It has also been attributed to the chronic effect of the oxidative stress associated with the underlying inflammatory process.[17] Mutations in genes regulating telomere maintenance have also, though rarely, been implicated.[34-37] In the current study, we provide evidence for age-inappropriate telomere loss in PB leukocytes of patients with CIN, an acquired disease entity characterized by immune-mediated suppression of neutrophil production by activated T lymphocytes and inhibitory cytokines in the BM.

Using a real time quantitative PCR method, we evaluated the relative telomere lengths of PB granulocytes and PBMCs, mainly consisting of lymphocytes and monocytes, in CIN patients and age-matched healthy individuals. A markedly significant decrease in relative telomere length was observed in both cell compartments of CIN patients compared to healthy controls, implying accelerated telom-

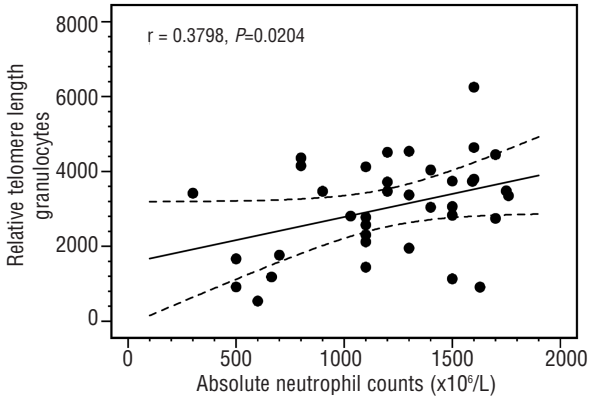

Figure 4. Correlation between granulocyte relative telomere values and absolute neutrophil counts in CIN. The regression line (± 95% confidence limits) show the correlation between individual relative telomere length values (estimated as relative telomere/single-copy-gene ratio values) and absolute neutrophil counts in the cohort of CIN patients studied. Coefficient of correlation (r) and degree of significance (P value), according to Spearman's correlation test, are indicated. CIN: chronic idiopathic neutropenia.

ere attrition in the hematopoietic stem cell compartment. The main cellular defect in CIN BM concerns primarily the committed $CD34^+/CD33^+$ myeloid progenitors.[3] However, a compensatory proliferation of the more primitive stem cells in order to increase the influx of cells in the more differentiated progenitor cell compartment cannot be excluded and might help explain the short telomeres obtained not only in the granulocytes but also in PBMCs.

However, mechanisms other than the increased stem/progenitor cell division rate may have determined the telomere length in patient PB cells. In particular, it has been shown that antigen-driven[38] or even mitogen-induced[39] activation of T lymphocytes is associated with accelerated telomere erosion resulting from the increased cellular proliferation rate.[27] Given that the main component of PBMCs is the T-cell fraction which is activated in CIN, we assume that an increased T-cell replicative history may also account for the inappropriate telomere shortening of patient PBMCs. In favor of this hypothesis is the increased expression of the Ki-67 nuclear antigen in the $CD4^+$ and $CD8^+$ cells of CIN patients suggestive of an intense proliferation rate of patient T cells.[28] Furthermore, the oxidative stress secondary to the underlying inflammatory process may also have accelerated the telomere loss in patient PB cells.[40] This mechanism has been implicated in telomere damage of hematopoietic cells in patients with AA and MDS.[41,42]

The ribonucleoprotein enzyme telomerase is involved in the maintenance of telomere length not only in germline and cancer cells but also in normal T and B lymphocytes and hematopoietic stem cells, albeit at low levels. However, telomerase activity increases upon lymphocyte activation and during the stem cell proliferation and differentiation process.[31] PB granulocytes and monocytes do not express telomerase activity.[31] To explore whether low telomerase activity may have a role in the abnormal telomere shortening of patient hematopoietic cells, we evaluated telomerase activity and hTERT gene expression in PB cells of CIN patients and healthy controls. As anticipated, no telomerase activity was identified in patient or normal granulocytes. In PBMCs, hTERT expression was identified in low but detectable levels in both patients and healthy controls with no significant differences. Telomerase activity, however, was identified at a significant lower frequency in the group of patients compared to controls. The reason for this difference is not clear given the age and sex compatibility between the two groups. Low telomerase activity has been associated with mutations in genes of telomerase complex in rare cases of apparently acquired AA. In particular, heterozygous mutations of the TERT gene have been described in these rare patients and probably impair telomerase activity by haploinsufficiency.[34] On the other hand, mutations of TERC which encodes the RNA component of telomerase complex have only been involved in the autosomal dominant cases of dyskeratosis congenital,[43,44] whereas genetic variations in the telomeric repeat binding factor 1 (TRF1) which binds to telomeric DNA have been associated with acquired AA but not with alterations in telomeres or telomerase activity.[35] A mutational analysis of TERT has not been performed in our patients. However, this possibility seems unlikely because none of the patients had clinical or laboratory evidence of AA and the available family history was also negative for AA. The possibility that the low telomerase activity in PBMCs of CIN patients is the result of premature immunosenescence associated with the replicative lymphocyte exhaustion rather than the cause of the telomere shortening seems more likely. The lack of any correlation between the telomerase activity or hTERT and relative telomere length of PBMCs in patients seems to support this hypothesis. The above mechanism has been described in patients with autoimmune and systemic immune-mediated diseases showing also accelerated telomere erosion in PBMCs.[45]

The relative telomere length of patient PBMCs, although lower than the healthy controls was, as expected, inversely correlated with patient age. In patient granulocytes, the anticipated inverse correlation between telomere length and age was abrogated, and the low relative telomere length in the entire group of CIN patients was actually due to the markedly decreased telomeres in the younger age groups. In patients over the age of 50 years, the relative telomere length of granulocytes was similar to the age-matched healthy individuals. We may hypothesize that, in younger patients, the $CD34^+/CD33^+$ cells show increased proliferative/compensatory potential compared to the older patients which is reflected by the shorter telomere lengths in their progeny. Interestingly, a lack of any significant decline in granulocyte telomere length with age has been observed in patients with AA and has been attributed to the increased proliferative potential of stem cells in younger subjects.[13] The lack of any significant age-dependent decline of telomeres in granulocytes of CIN patients may represent an additional mechanism for the absence of any statistical difference in telomere lengths between PBMCs and granulocytes in the older CIN age groups.

We did not identify any significant correlation between telomere length of granulocytes or PBMCs and clinical and laboratory parameters such as disease duration, white blood cell, lymphocyte or monocyte counts. However, a positive correlation was observed between the relative telomere length of granulocytes and absolute neutrophil counts, suggesting that the telomere length of the affected cell population essentially reflects the severity of neutropenia in CIN.

In conclusion, this is the first study to show that, similar to patients with immune-mediated BM disorders, CIN patients display age-inappropriate telomere shortening in PB hematopoietic cells. We have speculated that a compensatory increased proliferation of the BM hematopoietic stem/progenitor cells in association with the intense, activation-induced, lymphocyte replication mainly account for this abnormality. Further studies on BM hematopoietic stem/progenitor cell populations and on individual PB samples time-course, combined with studies for potential hTERT mutations, will help to delineate the underlying mechanisms for the observed telomere/telomerase alterations in CIN and their clinical relevance.

Authorship and Disclosures

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