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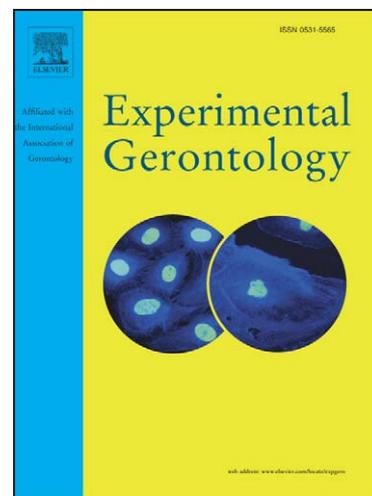
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Short Report**Telomere length-heterogeneity among myeloid cells is a predictor for chronological ageing**

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Telomere length, ageing, lymphocytes, granulocytes, monocytes

ABSTRACT

We have previously shown that mean telomere length (TL) of granulocytes reflects TL of myeloid bone marrow cells extremely well. Here we analysed the distribution of TL from peripheral blood granulocytes and lymphocytes in 61 female and 68 age-matched male healthy volunteers. We show that the age-dependent decline in TL occurs more rapid in peripheral blood lymphocytes (53 bp/year) than in granulocytes (39 bp/year; $p < 0.001$), while women having longer telomeres than men. We also observed the best correlation for age and telomere length to be present in lymphocytes. The coefficient variation (CV) of TL distribution in granulocytes and monocytes was significantly higher in older compared to younger individuals, indicating an increase in telomere length heterogeneity of myeloid cells and their bone marrow residing precursors during ageing. In a multivariate statistical model, CV and lymphocyte TL were able to explain 50% of chronological ageing.

1. Introduction

Telomeres, hexameric TTAGGG DNA repeats the ends of chromosomes, are widely regarded as the internal biological clock of a living organism, and shorten by a few base pairs with every cell division. In addition, they are also crucial to maintaining chromosomal integrity (Blasco, 2005). Age-related pathologies with short telomeres include infertility, impaired wound-healing and liver cirrhosis. Individuals with shorter leukocyte telomeres have a significantly higher risk of developing coronary heart disease or of dying from cardiovascular and infectious disease (Brouillette et al., 2007; Cawthon et al., 2003). We have published recently that telomere length from granulocytes correlates extremely well with their bone marrow residing myeloid precursors ($r = 0.94$, $p = 0.001$), justifying the quantification of TL from peripheral blood granulocytes as an indicator of stem cell divisions (Spyridopoulos et al., 2008). The enzyme telomerase counteracts telomere loss in proliferating cells and extends their capacity for replication. Human embryonic stem cells express the telomerase reverse transcriptase (TERT) gene and demonstrate telomerase activity. TERT has an important role in the maintenance of human ESC pluripotency, cell cycle regulation, and in vitro differentiation capacity (Saretzki et al., 2008; Yang et al., 2008). Unlike in germ cells, replication of hematopoietic stem cells leads to telomere shortening and - in the long term - to the subsequent exhaustion of proliferative capacity. It remains unclear which factors determine telomere shortening in vivo. The goal of this study was to investigate which telomere-associated parameters in myeloid and lymphoid leukocyte subpopulations best determine chronological ageing. For this, we used the flow FISH technique which enabled us to determine TL in different cell populations as well as the heterogeneity of TL distribution in individual volunteers.

2. Materials and methods

2.1 Study population

Peripheral blood was collected from 129 healthy volunteers without any past medical history. All individuals had to be physically fit and non-obese, walking or jogging at least 20 km / week. Active smokers or individuals on any kind of medical treatment were also excluded from the study. For further studies on specific myeloid cell subpopulations, we used additional 27 male healthy volunteers (14 young and 13 old). All studies have been performed under approval by our institutional ethics board and informed consent was obtained from all volunteers.

2.2 Isolation of peripheral blood cells

Red blood cells from EDTA blood were lysed by incubating the pelleted cells twice with ammonium chloride solution (Stem-Cell Technologies), each time in a 1:4 ratio for 10 minutes on ice. Nucleated cells were recovered by centrifugation. CD14⁺ monocytes were isolated from peripheral blood mononuclear cells by Ficoll density gradient centrifugation, followed by immunomagnetic bead sorting (Milteny Biotech, Gladbach, Germany) according to the manufacturer's protocol.

2.3 Measurement of telomere length (Flow-FISH)

Telomere length measurements were carried out as previously described. (Baerlocher et al., 2006; Spyridopoulos et al., 2008; Spyridopoulos et al., 2004) In brief, each sample, containing 100,000 bovine thymocytes as an internal standard and 200,000 cells from the donor, was resuspended in a hybridization mixture containing telomere-specific N-terminal FITC-conjugated (C₃TA₂)₃ PNA probe, washed and counterstained with propidium iodide prior to FACS analysis. Telomere length measurement in CD15⁺ granulocytes was performed by Multicolor Flow-FISH following whole blood lysis.

2.4 Statistics

Data are reported as median and interquartile ranges [IQR] if not stated otherwise. Correlations between age and telomere-related parameters were performed with the use of Spearman rank coefficient. For stepwise multivariate regression analysis, we retained all variables with *p*-values < 0.05 from the univariate analysis. We excluded variables with missing values in a pairwise procedure. Tests were two sided and *p*-values < 0.05 regarded statistically significant. All statistical tests were performed using SPSS Version 15.0 for Windows (SPSS Inc., Chicago, USA). For comparison of correlating *r*'s Steiger's and Fishers's Z-test Dr. Calvin P. Garbins software (FZT.exe) was used (www-class.unl.edu/)

psycrs/statpage/comp.html).

3. Results

Characteristics of 129 healthy volunteers are shown in Table 1. Men and women were age-matched with a median age of 41 and 40 years, respectively. HDL cholesterol was as expected higher in female compared to male volunteers ($p=0.039$) while LDL, triglycerides and HbA1c were equally distributed among both genders. Other cardiovascular risk factors such as hypertension and smoking were excluded a priori. We measured mean telomere length (TL) of peripheral granulocytes and lymphocytes by the Flow-FISH method, which also allows evaluation of telomere length distribution. Interestingly, men had significantly shorter telomere length in lymphocytes than women (median 6.6 kbp vs. 7.6 kbp; $p=0.03$), while granulocyte telomeres did not differ significantly (Table 1). The coefficient variation (CV) of telomere fluorescence histograms in lymphocytes and granulocytes, which represents telomere length heterogeneity of either population in a single individual, did not differ between genders. We found a strong correlation between lymphocyte telomere length and chronological age for the whole population (Spearman rank correlation -0.601 vs. -0.501 in granulocytes, Table 2). This difference remained stronger in lymphocytes when analysing male and female volunteers separately.

To further investigate TL heterogeneity, we analysed the relation between the CV of TL and age. Surprisingly, CV of granulocytes showed a strong correlation with age (Spearman rank correlation 0.452, $p<0.001$), while lymphocyte CV correlated rather weak with age (Spearman rank correlation 0.279, Table 2). As expected, we also found an age-dependent decrease in leukocyte TL with a more rapid decrease of TL in peripheral blood lymphocytes (53 bp/year) compared to granulocytes (39 bp/year) (Figure 1A). This difference was statistically significant ($p<0.0001$). The correlation between granulocyte CV and age was strongest in male volunteers (Spearman rank correlation 0.50, $p<0.0001$, Figure 1B), which prompted us to conduct further experiments on myeloid cells in an additional male population, consisting of 14 healthy young volunteers (mean age 25 years) and 13 older healthy volunteers (mean age 65 years). To acquire even more specific data on CV of myeloid cells, we analysed immunomagnetically isolated CD14⁺ monocytes (purity over 95% as confirmed by flow cytometry) and surface-stained CD15⁺ granulocytes using standard Flow-FISH or Multicolor Flow-FISH, respectively. Here, we found that CV differed significantly between young and old healthy volunteers, both for monocytes (12% vs. 14%, $p<0.001$; Figure 1C) and granulocytes (12% vs. 14%, $p<0.001$; Figure 1C).

Finally, multivariate regression analysis revealed that both, CV of granulocytes and TL of lymphocytes were independent predictors of age (data not shown). In this model, the combination of these 2 parameters explained 50% of chronological ageing in our population ($p < 0.001$).

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4. Discussion

Our data show for the first time that lymphocyte TL decreases significantly faster and, most importantly, correlates better with age than granulocyte TL. The latter is most likely related to the compartment of memory lymphocytes. Since memory T cells contain much shorter telomeres (Rufer et al., 1999), their increasing number with age adds quicker to the decrease in lymphocyte TL. Granulocytes, on the other hand, shorten at the same rate as naïve T cells (Rufer et al., 1999). Our findings are also consistent with previous work from others and our own data, demonstrating a similar decline in telomere length in total lymphocytes from adult volunteers.

Furthermore, we found women to have significantly longer telomeres in the lymphocyte population compared to the male population. The difference of 365 base pairs would correspond to a life span of approximately 6 years. One possible explanation for this finding is the expression of the estrogen receptor in T lymphocytes. In fact, treatment of T cells with estrogen enhances telomerase activity in these cells (Effros et al., 2005). Guan and coworkers found that in the Japanese population, until the age of 50, telomere length decreases faster in men compared to women (Guan et al., 2007). This ratio was reversed in postmenopausal women.

Finally, we detected an age-dependent increase in telomere length distribution among myeloid peripheral blood cells. Previously it has only been described that telomere length variation in a single individual is more pronounced in lymphocytes than in granulocytes (Rufer et al., 1999), which is intuitive, given the difference in TL between naïve and memory lymphocytes. The latter are almost 2000 base pairs shorter, independent of age. The novel and therefore surprising finding in our cohort though was that TL heterogeneity of myeloid cells increased with age as well, yet this effect was more pronounced in male volunteers. To exclude that this could have been a technical issue attributed to the identification of granulocytes by their scatter properties in flow cytometry, we chose to extend our experiments with an additional study population, consisting of 14 young and 13 old healthy male volunteers. This time we used immunomagnetic bead sorting as well as Multicolor Flow-FISH to specifically analyse CD14⁺ monocytes and CD15⁺ granulocytes based on their surface antigen properties. The findings in both cell populations confirmed our initial results, in that TL heterogeneity in myeloid cells increases with age.

What is the relevance of such a finding? We have published previously that TL of granulocytes reflects TL of myeloid bone marrow precursors extremely well (Spearman rank

correlation greater 0.95) (Spyridopoulos et al., 2008). Together, our results strongly suggest that TL heterogeneity of bone marrow residing precursors increases with age. Therefore individuals with the same mean TL but higher TL heterogeneity will have both some shorter and some longer telomeres. This implies that a proportion of myeloid precursors and stem cells might reach critical telomere length faster, which could potentially contribute to the exhaustion of bone marrow function (Kissel et al., 2007). Although two other studies have shown that shorter fragments of telomeres increase with ageing, these findings were raised in total leukocyte populations (Guan et al., 2007; Kimura et al., 2008), which apparently also include the very short telomeres from CD4 and CD8 memory lymphocytes. Kimura and coworkers have also shown that the association of mortality with mean leukocyte TL was much weaker than the association with shorter telomeres. Telomere length distribution of leukocytes was a better predictor of mortality. Prospective studies will have to show whether TL in specific leukocyte subpopulations or TL heterogeneity in myeloid cells will demonstrate a better correlation with markers of cardiovascular disease as compared to the commonly used leukocyte TL.

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Legend Figure 1:

A. Mean telomere length (TL) for granulocytes (●) and lymphocytes (▲) is plotted against age. Linear regression curves for the granulocyte (---) and lymphocyte (—) population are shown. Slopes for the age-dependent decline in telomere length were 39 bp/year for the granulocyte population compared to 53 bp/year for the lymphocyte population, respectively. Goodness of fit (r value) and p value for slope different from zero are indicated for the granulocyte and lymphocyte regression curves, respectively. **B.** Coefficient variation (CV) of granulocytes is plotted for the male study population (n=68) against age. **C.** Box plots (quartiles) are presented for CV of CD14⁺ monocytes and CD15⁺ granulocytes from either male young healthy volunteers (mean age 26.7±1.8 years; n=14) or older male healthy volunteers (mean age 65.1±2.1 years; n=13).

Table 1: Characteristics of study population

	Female volunteers (n=61)	Male volunteers (n=68)	
	median (IQR)	median (IQR)	p
Age (years)	40 (28;54)	41 (30;60)	0.165
HDL cholesterol (mg/dl)	68 (57;86)	57 (46;68)	0.039
LDL cholesterol (mg/dl)	101 (88;130)	108 (80;129)	0.969
Triglycerids (mg/dl)	112 (78;157)	106 (78;200)	0.410
HbA _{1c} (%)	5.3 (5.0;5.6)	5.3 (5.0;5.7)	0.851
TL WBC (10 ³ bp)	7.8 (6.6;8.8)	7.3 (6.3;8.4)	0.092
TL granulocytes (10 ³ bp)	7.8 (6.9;8.9)	7.4 (6.6;8.5)	0.157
TL lymphocytes (10 ³ bp)	7.6 (6.2;8.6)	6.6 (5.8;7.9)	0.030
ΔTEL (gran-lymph) (10 ³ bp)	0.4 (0.1;1.0)	0.7 (0.4;1.1)	0.025
CV WBC (%)	13.9 (12.7;18.5)	15.6 (12.8;19.0)	0.338
CV granulocytes (%)	10.6 (10.1;11.4)	10.9 (10.3;11.8)	0.129
CV lymphocytes (%)	21.4 (18.2;23.6)	21.2 (18.4;23.2)	0.341

IQR = interquartile range, ΔTEL (Gr-Ly) the intraindividual base pair difference between granulocytes and lymphocytes

Table 2: Spearman rank correlation of telomere length parameters vs. age

	r (vs. age)	r (vs. age)	r (vs. age)
Parameter	Female (n=61)	Male (n=68)	All (n=129)
TL white blood cells [bp]	-0.478**	-0.550**	-0.526**
TL granulocytes [bp]	-0.452**	-0.528**	-0.501**
TL lymphocytes [bp]	-0.550**	-0.635**	-0.601**
ΔTEL (Gr-Ly) [bp]	0.375*	0.417**	0.403**
CV granulocytes (%)	0.356*	0.502**	0.452**
CV lymphocytes (%)	0.279 [§]	0.331*	0.279*

significance levels: [§]p<0.05, *p<0.01, ** p<0.001. CV indicates coefficient variation, ΔTEL (Gr-Ly) the intraindividual base pair difference between granulocytes and lymphocytes

Table 3: Linear regression analysis: Dependent variable age (n=129)

age (years)	b	SE	β	T	p
constant	49.0	15.1	-	3.254	0.001
mTL of lymphocytes [10^3 bp]	-0.468	0.826	-0.466	-5.666	<0.001
CV granulocytes (%)	2.528	1.019	0.204	2.481	0.014

Multiple regression analysis predicting age from telomere length and CV. All variables which correlated in the univariate analysis ($p < 0.05$) were included into the multivariate analysis. Unstandardized coefficient (b), standard error of b (SE), standardized coefficient (β), T, and multivariate p values of each variable are listed. Other variables included into this analysis were gender, mTL of granulocytes, mTL of leukocytes, CV of lymphocytes, CV of WBCs.

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Figure 1

