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No Telomere Shortening in Marrow Stroma from Patients with MDS

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Abstract

Telomere shortening with age may lead to genomic instability and an increased risk of cancer. Given the role of the microenvironment in the pathophysiology of the myelodysplastic syndrome (MDS), primarily a disease of older age, we determined telomere length in primary cultured marrow stroma cells using quantitative-fluorescence *in situ* hybridization (q-FISH) and quantitative-PCR (q-PCR). qFISH showed comparable rates of decrease in telomere length with age in MDS patients and age-matched healthy controls. Telomere length assessment by qPCR showed similar results. These findings suggest a lack of significant differences between MDS patients and healthy controls in terms of telomere stability in marrow stroma in contrast to that observed in hematopoietic cells. In conclusion, this demonstrates that although MDS stroma cells and hematopoietic cells share the same microenvironment, the stromal cells do not share the processes that contribute to accelerated telomere attrition, suggesting that stromal cell proliferative potential is not limiting in MDS.

Keywords

marrow stroma; telomere; stroma; qFITC

INTRODUCTION

Telomeres, the region of repetitive DNA sequences (TTAGGG) at the end of each chromosome provide stability and protect chromosomes from end-to-end fusions, degradation, and recombination. Telomere attrition exposes chromosome ends, activates cell cycle checkpoints and cellular senescence, and, in the absence of proficient cell cycle checkpoints, promotes cycles of bridge-breakage-fusion [14]. The pronounced age-related decline in telomere length may promote genetic instability and increase the risk of malignancy [1]. The incidence of myelodysplastic syndrome (MDS) increases progressively with age, and abnormal shortening

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Authors' contributions. Mario Marcondes designed the experiments, conducted the experiments and wrote the manuscript.
Steve Bair contributed to the analysis.
Peter Rabinovitch provided reagents, made suggestions to the experimental design, assisted in interpreting the results, and revised the manuscript.
Ted Gooley carried out the statistical analysis.
H. Joachim Deeg provided marrow samples, assisted in analyzing the results and provided revisions to the manuscript.
Rosana Risques contributed technical expertise.

of telomeres has been shown in hematopoietic cells from patients with MDS [5,6,16–18,20, 22].

The role of the marrow microenvironment in the pathophysiology of clonal marrow disorders such as MDS remains controversial [7,23]. While macrophages, for example, are part of the hematopoietic clone, stroma cells are non-clonal [2]. However, several investigators have reported dysfunction of MDS-derived stroma [7], and our own work shows abnormal expression of cytokines and receptors in marrow stroma from patients with MDS [13]. Conversely, hematopoietic cell transplantation from allogeneic donors is generally successful [21], although a recent report support suggests slower kinetics than in other diseases [4].

To further characterize marrow stroma from patients with MDS, we investigated telomere length in marrow fibroblasts as one parameter of integrity of the MDS marrow microenvironment.

MATERIALS AND METHODS

Patients and controls

Fresh bone marrow aspirates were obtained from patients with all subtypes of MDS, including one patient with myelomonocytic leukemia (CMML) (n=38). Patients were 42 to 88 years old, 24 were male and 14 female. In addition, samples were obtained from 13 healthy volunteers, 35–80 years of age. All patients and healthy volunteers had given informed consent as required by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Cell lines and culture

HS5 is a human marrow stroma cell line that has been extensively characterized [8,9,11,24]. HS5 cells were maintained in complete medium (RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum [FBS], 1% glutamine, and 1% sodium pyruvate) and propagated at 37°C in a 5% $C0_2$/air atmosphere. Before experimental manipulation, cells were sedimented at 1200 rpm (300 g) and resuspended in complete medium and aliquoted in 6-well plates at 10^6 cells/mL.

Culture, isolation and purification of bone marrow stroma cells

Bone marrow mononuclear cells (BMMC) were isolated as described [13]. Approximately 25 × 10^6 cells were incubated in 75-ml cell culture flasks using non-hematopoietic expansion medium (Miltenyi Biotec) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced weekly, and non-attached cells were discarded. When adherent cells reached 70% confluence, cultures were split for further propagation or used for studies. Population doubling times were determined weekly for 7 weeks.

In preparation for studies, adherent cells were washed with phosphate-buffered saline (PBS), trypsinized (0.25%) for 10 minutes, and analyzed by flow cytometry to detect and exclude possible myeloid/lymphoid cell contamination. The antibody specificities used for identification of myeloid/lymphoid cells included CD45, CD11b, CD14 and CD34. To phenotypically characterize adherent (“stroma”) cells, we used antibodies for CD54, CD73, CD90, and CD177, obtained from BD Biosciences (San Jose, CA, USA) and R&D Systems (Minneapolis, MN, USA). After phenotypic characterization, highly purified stroma cells were plated in 6-well plates containing glass-covered slides coated with Poly-(Lys) (Invitrogen, La Jolla, CA). After reaching 75% confluence, those cells were fixed for 30 min in 4% paraformaldehyde at room temperature and washed 3 times in PBS. Slides were air-dried and frozen at −80°C, and subsequently analyzed using fluorescent in situ hybridization (FISH).

Genomic DNA was extracted from parallel cell samples using the Qiamp DNA extraction kit (Qiagen,Hilden, Germany) following the manufacturer's instructions.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained from blood drawn into heparinized syringes by separation over a Ficoll hypaque gradient as described [12]. DNA was extracted as described for stroma cells above.

Quantitative analysis of telomeres using (q-FISH) and (q-PCR)

Dual-color (telomere and centromere) q-FISH was performed as previously described using confocal microscopy and image analysis [15]. A minimum of 50 stroma cells was analyzed per case to obtain an average epithelial/stromal telomere intensity (proportional to telomere length), which was calculated as the epithelial/stromal ratio [15].

Telomere and centromere q-FISH was also performed on aliquots of the HS5 cell line as an internal control, and these values were used for inter-experimental normalization. The intra and inter-assay variability (CV) for the normalized q-FISH was 0.1087 (10%).

Following genomic DNA extraction as described above, telomere length was measured by q-PCR. Briefly, each sample was amplified for telomeric DNA and for 36B4, a single-copy control gene that provided an internal control to normalize the starting amount of DNA. Two control samples were included in each experiment to allow for normalization, and periodic reproducibility experiments were carried out to confirm correct measurements. The intra-assay and inter-assay variabilities (coefficient of variation) for quantitative PCR were 6% and 7%, respectively. Because the *T/S* ratio is a relative measure of telomere length, the mean of *T/S* ratio of the cohort was normalized to 1.0 to facilitate comparisons.

Statistical analysis

Linear regression was used to evaluate the relationship between telomere length and age and between telomere length and doubling time of stroma cells in culture. The slopes of the regression lines were compared between MDS patients and healthy volunteers by fitting an interaction term between the slope and group. Comparison of mean telomere lengths between MDS patients and healthy volunteers was performed with the two-sided *t* test. Telomere lengths were compared between multiple diagnostic subgroups among the MDS patients using analysis of variance.

RESULTS AND DISCUSSION

We compared telomere length in stroma cells from 38 patients with MDS to 13 healthy controls in the same age bracket. The centromere and DNA probes served as internal controls for telomere brightness that might have resulted from reduced accessibility of the telomere probe. To control for inter-experiment differences in sample fixation, processing, and staining, data are expressed as the ratio of telomere-to-DNA cell fluorescence divided by the average telomere-to-DNA cell fluorescence of the HS5 cell line that was included in every experiment. Telomere length shortening in MDS patients measured by q-FISH was not statistically significantly different from that in age matched controls (mean MDS=0.96, standard deviation [SD]=0.23; mean control= 1.03,SD=0.28, p=.39, 95% confidence interval for difference in telomere length, - 0.2283 to 0.091). While there was considerable variation in telomere length between individuals with MDS, telomere lengths progressively declined with age in both MDS *and* control groups (R=−0.36, slope=−0.008) (each increase in age of one year associated with decrease in telomere length of 0.008); and R=-0.55, slope=-0.0079, respectively (p=.99, interaction test; Figure 1A). A second technique, q-PCR, also failed to show a substantial

difference in the association between telomere length and age between patients with MDS and controls (data not shown).

The doubling times of stroma cells were similarly correlated with telomere length (R=0.36; p=0.02), and as shown in Figure 2, the slopes for population doubling were similar for patients with MDS and healthy individuals (p=0.5), as would be expected in view of comparable telomere length.

Others have indicated a common pattern of telomere length shortening with age in many different tissues, at a rate of 100 to 150 base pairs/year [3], or approximately 0.08% annually. Our findings are consistent with those previous results (Figure 1B). When IPSS was treated as a continuous linear variable, the correlation with telomere length was not statistically significant (p=0.83), and a trend test on IPSS as categorized in Figure 1D yielded p=−0.78. Further, there was no statistically significant difference in telomere length between the seven diagnostic groups as categorized in Figure 1C (p=0.13), although the number of patients in these groups was small, thereby limiting the power to detect a difference. Results of q-FISH, karyotyping and stratification by IPSS [10] are shown in Table 1.

Finally, to place our data into the context of previously published results on telomere shortening in cells derived from the hematopoietic clone [5,6,17,18,22], we carried out a comparison of telomere length in stroma to telomere length in PBMC, which were available in two MDS patients and one healthy donor. The ratio of PBMC to stroma telomere length as determined by qPCR was 0.7 and 0.6 in the two patients, respectively, and 1.2 in the healthy donor. The telomere length in PBMC in the healthy donor was exactly in the range published by R.A. Risques et al. [19], while the lengths were 8 to 10-fold shorter in the two MDS patients.

Thus, these data provide no evidence for accelerated telomere shortening in the marrow stroma of patients with MDS beyond what is expected among healthy volunteers. While the findings do not exclude a role for stroma in the pathophysiology of MDS, they argue strongly against enhanced turn-over or genetic instability in those cells. We have not excluded the possibility that telomere shortening was counter-acted by increased telomerase activity. However, the most likely explanation for the present results is that MDS marrow stroma cells are not clone-derived, and that in spite of sharing the microenvironment with hematopoietic cells, they are not subject to the combination of increased proliferation or oxidative stress that presumably underlies the telomere attrition of MDS hematopoietic cells previously reported [5,6,16–18, 20,22].

In summary, these data show that marrow stroma from patients with MDS undergoes telomere shortening only at a rate that corresponds to patient age [22], and that any abnormality of stroma function in MDS more likely occurs in response to signals derived from the hematopoietic clone.

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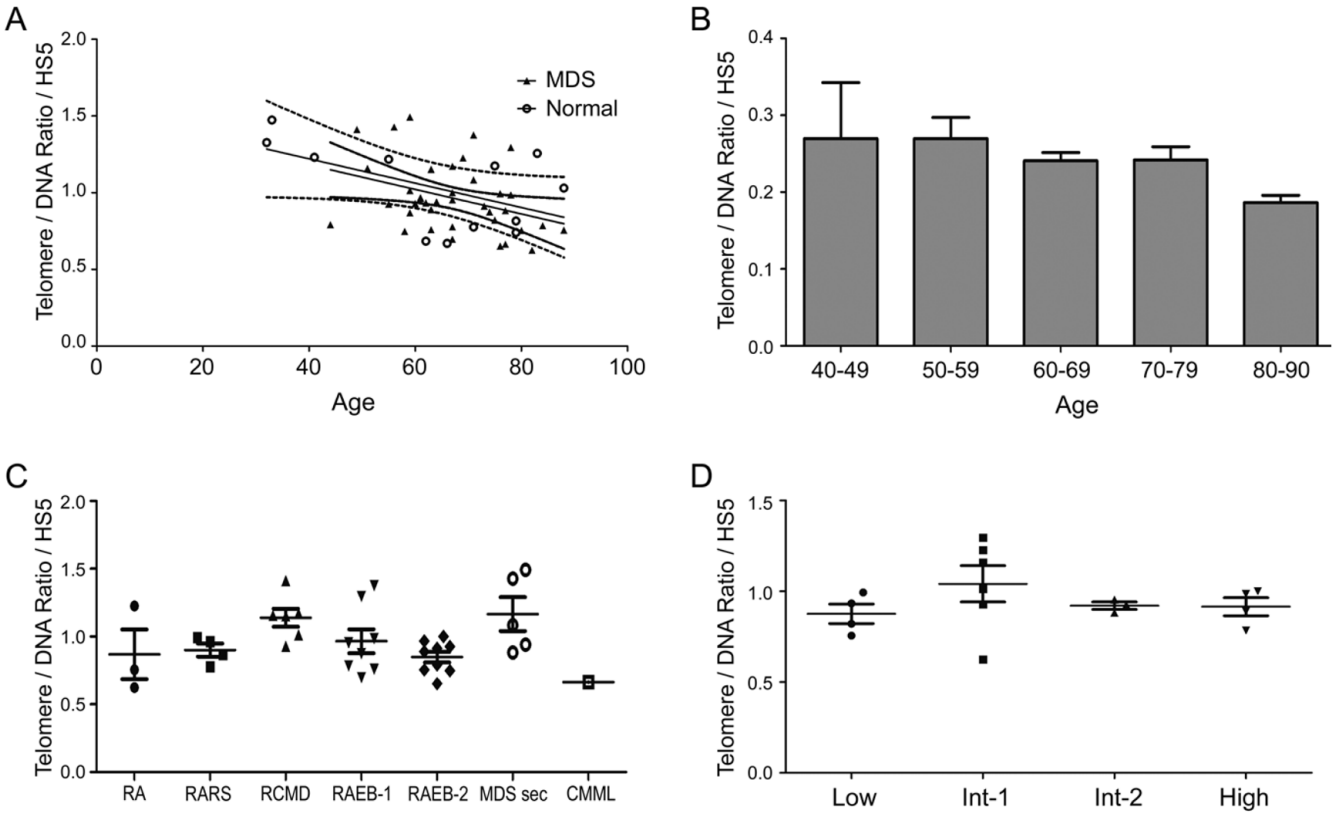

Figure 1. A) Telomere shortening in normal and MDS marrow stroma

Telomere length (by q-FISH) decreased progressively with age for both patients with MDS (broken curves; R= −0.36, slope= −16) and controls (solid curves; R= −0.55, slope= −38, .p=0.15). **B) Telomere shortening in patients with MDS.** Decrease by 0.08% of total length per year. **C) Telomere length by MDS category.** There was no correlation between telomere length and MDS category (RA= refractory anemia; RARS= RA with ring sideroblasts; RCMD+ refractory cytopenia with multilineage dysplasia; RAEB = RA with excess blasts [1 =5–9%; 2 = 10–19%]; MDS sec = treatment related MDS; CMML = chronic myelomonocytic leukemia**). D) Telomere length and IPSS score.** There was no correlation between IPSS category [10]and telomere length.

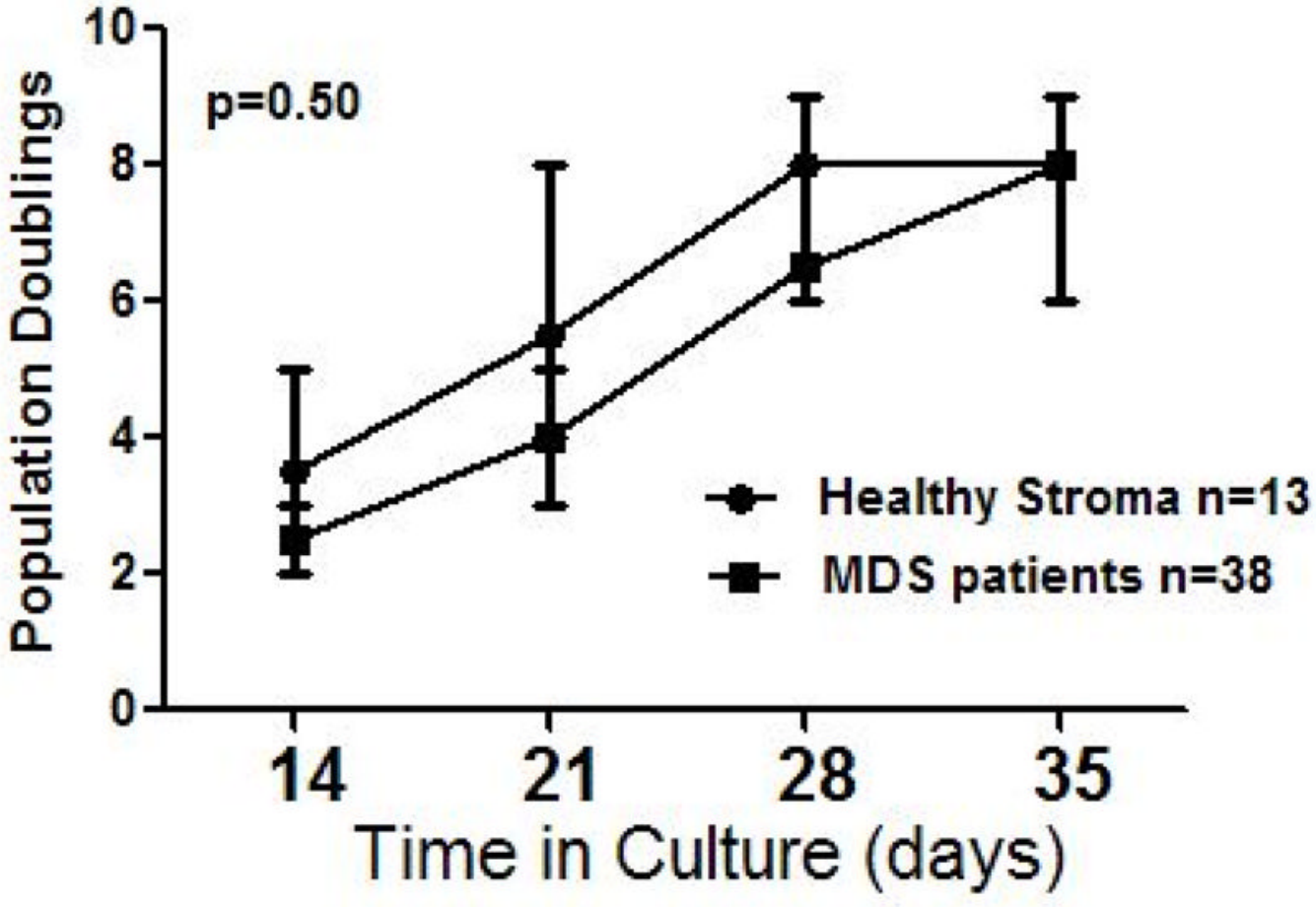

Figure 2. Population doubling times of marrow stroma from healthy donors and MDS patients
Slopes for patients and healthy controls were similar (p=0.50).

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Table 1

Clinical Variables of MDS Patients and Telomere Size

Diagnosis	Chemotherapeutic Treatment	Age	Cytogenetic Markers	IPSS	BM Cellularity	Telomere/DNA/HS5 DNA
RA	Untreated	69	Normal	0.5	hyper	1.2266
RA	Untreated	82	Normal	0.5	normal	0.6244
RA	Untreated	88	Normal	0.0	normal	0.7561
RARS	ATG and etanercept	59	Normal	0.5	hyper	0.8689
RARS	Etanercept and 5-azacytidine	61	Normal	N/A	hyper	0.9637
RARS	Lenalidomide	67	Normal	N/A	hyper	0.7773
RARS	Untreated	76	Normal	0.0	normal	0.9935
RCMD-RS	Untreated	60	Normal	0.5	hyper	0.9290
RCMD	Thalidomide	63	t(2:11)	1.0	hyper	1.1520
RCMD	Untreated	49	Complex, del(7)	1.5	hypo	1.4119
RCMD	Untreated	51	Normal	0.5	normal	1.1578
RCMD	Untreated	59	Normal	0.5	normal	1.0133
RCMD	Etanercept	67	tri(8),del(7)	1.0	normal	1.1715
RAEB-1	Filgastrim and epoetin beta	67	Normal	2.0	hyper	0.9538
RAEB-1	Untreated	78	Complex	2.5	hyper	0.9852
RAEB-1	Untreated	78	Normal	1.0	hyper	1.2953
RAEB-1	Untreated	84	del7	2.5	hypo	0.7844
RAEB-1	Etanercept and 5-azacytidine	63	Normal	0.0	normal	0.7584
RAEB-1	Etanercept and 5-azacytidine	67	Complex	1.5	normal	0.6967
RAEB-1	Etanercept and 5-azacytidine	71	Complex, del(7)	2.5	normal	1.3762
RAEB-1	Etanercept and 5-azacytidine	74	Normal	2.0	normal	0.8742
RAEB-2	7+3	44	Normal	0.5	hyper	0.7911
RAEB-2	7+3 and 5-azacytidine	61	Normal	2.5	hyper	0.9675
RAEB-2	Untreated	63	Complex, del(7)	3.0	hyper	0.8900
RAEB-2	Untreated	67	Complex	3.0	hyper	1.0004
RAEB-2	Untreated	76	t(6:9)	2.5	hyper	0.6525
RAEB-2	Etanercept and 5-azacytidine	80	Normal	1.0	hyper	0.7538
RAEB-2	Etanercept and 5-azacytidine	73	del(5q)	1.0	hypo	0.9119
RAEB-2	Untreated	55	t(10:22)	1.5	normal	0.9264
RAEB-2	5-azacytidine, thalidomide	58	tri(21)	0.5	normal	0.7469

Diagnosis	Chemotherapeutic Treatment	Age	Cytogenetic Markers	IPSS	BM Cellularity	Telomere/DNA/HS5 DNA
MDS - 5q(-)	Untreated	62	del(5q)	0.0	normal	0.9332
MDS-unclassified	Untreated	75	Normal	0.0	normal	0.8211
MDS sec	FLAM	64	Normal	N/A	hyper	0.9402
MDS sec	Untreated	77	add(11q)	1.5	hyper	0.8824
MDS sec	7+3	56	Normal	N/A	hypo	1.4281
MDS sec	Irradiation	59	del(13)	0.5	normal	1.4933
MDS sec	Clofarabine	71	tri(8),del(7)	N/A	normal	1.0847
CMML	Untreated	77	Normal	N/A	normal	0.6641