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Lupus 2007; 16; 955
DOI: 10.1177/0961203307084299

Lupus (2007) 16, 955–962
http://lup.sagepub.com

PAPER

Telomere length analysis in monocytes and lymphocytes from patients with systemic lupus erythematosus using multi-color flow-FISH

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In order to analyse telomere length in subsets of human peripheral blood lymphocytes and monocytes, we modified a recently developed multicolor flow- fluorescent in situ hybridization (FISH) methodology that combines flow-FISH and antibody staining for cell surface antigens. We analysed telomere length of peripheral blood mononuclear cells in a group of 22 patients with systemic lupus erythematosus (SLE) and 20 age-matched healthy donors. We found that neither CD4+, CD8+, CD19+ cells nor CD14+ monocytes have significantly shorter telomeres compared with their healthy counterparts. On the basis of these findings, we then used monocyte telomere length as internal reference in order to control for intra-individual variability in telomere length. By using this approach, we could demonstrate significant telomere shortening in all three lymphocyte subsets (in all cases $P < 0.05$) compared with monocytes. However, these differences did not vary significantly between SLE patients and controls. In summary, telomere lengths in subpopulations of hematopoietic cells can be monitored in patients with SLE using multicolor flow-FISH. While confirming data by other groups on telomere length in lymphocyte subpopulations, our data argue against an increased proliferation rate of peripheral blood monocytes reflected by accelerated telomere shortening in patients with SLE. *Lupus* (2007) **16**, 955–962.

Key words: flow-FISH; monocyte; systemic lupus erythematosus; telomere; telomerase

Introduction

Telomeres represent the natural end of the chromosomes. They consist of repetitive DNA sequences thereby acting as a protective cap to avoid chromosomal instability.[1] Owing to the so called 'end replication problem', telomeres shorten with each cell division unless they express the enzyme telomerase which is capable of maintaining telomere length. If cells reach a critical telomere length, cellular senescence and cell cycle arrest or apoptosis result.[2,3] As telomeres shorten with each cell division, telomere lengths reflect the proliferative potential and the replicative history of somatic cells.[4] Furthermore, accelerated telomere shortening has been implicated to be associated with genetic instability of cells and as such, suspected to be involved in the pathogenesis and clinical progression of a variety of hematological disorders (reviewed in[5]).

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production, lymphocyte hyperactivity and complement activation.[6] These features of an aberrant immune response lead to a wide spectrum of clinical symptoms. The pathogenesis of the disease remains unknown, but previous studies have shown an abnormal T-cell function. The T-cells in SLE patients show a diminished *in vitro* response to different stimuli and a defective response to IL-2.[7] Furthermore, a B-cell hyperactivity presumably mediated by T-cells and a production of pathogenic autoantibodies can be found.[8,9] Monocytes have long been thought to play a central role in the stimulation of the (auto-)immunity in SLE. Monocytes from SLE patients (or induced by SLE serum) have been shown to induce strong mixed lymphocyte reactions *in vitro*.[10] It has been hypothesized that interferon-alpha produced by plasmacytoid

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Received 8 March 2007; accepted 28 August 2007

10.1177/0961203307084299

dendritic cells (pDC) induces monocytes to differentiate into DC, which themselves are able to efficiently present self-antigens captured from apoptotic cells to B- and T-cells thus triggering and sustaining the autoimmune response (reviewed in[11]).

Previous studies of other rheumatic diseases, such as rheumatoid arthiritis (RA) showed telomere shortening in defined lymphocyte subpopulations suggesting an involvement in disease pathogenesis.[12–14] Furthermore, several studies investigated telomere length dynamics in more or less well-defined subpopulations of peripheral blood cells from SLE patients.[15–20]

Various methods exist to measure telomere length. The most common are southern blot analysis and flow-FISH, the latter combining fluorescent in situ hybridization (FISH) technique with flow cytometry.[21] Although southern blot represents the widely accepted standard method for telomere length measurement by analysing telomere restriction fragments (TRF), the flow-FISH methodology, especially multicolor flow-FISH, recently became increasingly important for several reasons: First, as opposed to southern blot and conventional flow-FISH, magnetic bead separation of phenotypically defined subpopulations of peripheral blood mononuclear cells (PMNC) is not requested, which is beneficial particularly for the analysis of rare cell populations. Secondly, together with conventional flow FISH and as compared with southern blot, measurement is no longer compromized by 'contaminating' subtelomeric DNA. Furthermore, the inclusion of cow thymocytes as internal control cells in each individual tube[22,23] allows for sufficient correction of intra- and inter-experimental variability in hybridization efficiencies between samples. Finally, in contrast to the southern blot methodology, the multicolor flow-FISH needs substantially fewer cells (in the order of 10^5 cells) and is less time-consuming thus allowing the analysis of sample sizes typically required in clinical studies.

In the current study, we therefore used a recently developed combination of flow-FISH and cell surface staining (multicolor flow-FISH) in order to investigate telomere length in leukocyte subpopulations of patients with SLE compared with healthy donors with particular focus on monocytes.

Material and methods

Patients

Peripheral blood samples of 22 SLE patients and 20 healthy donors (HD) were analysed in cooperation with the University of Erlangen. All experiments were approved by the ethic committees of the University of Tübingen and Erlangen and informed consent from HD was obtained according to the declaration of Helsinki. SLE was diagnosed based on the criteria of the American College of Rheumatology. Mean age ($\pm$SD) of the SLE patients and controls was 43.0 $\pm$ 15.0 years and 40.5 $\pm$ 15.1 years, respectively. Concerning the clinical characteristics of the 22 SLE patients, the following parameters were collected retrospectively: disease duration and treatment, total leucocyte count, absolute and relative lymphocyte count, platelet count, hemoglobin level, erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), serum neopterin level as well as serum complement level (C3 and C4). Owing to the small number of patients with collected CH50 levels and anti-dsDNA antibody titers, we decided to use a modified SLEDAI index to evaluate the disease activity.

Multi-color flow-FISH

Peripheral blood mononuclear cells of the SLE patients were isolated using Ficoll-Hypaque density gradient centrifugation (Biochrom, Germany) and cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma Aldrich, Germany) as reported previously[24] and stored in liquid nitrogen until use.

Peripheral blood leukocytes from HD were isolated using ammoniumchlorid (StemCell Technologies, Vancouver, Canada) and directly processed on the basis of the following protocol: Aliquots of 2×10^5 PBMC and 1×10^5 cow thymocytes were stained with biotinylated antibodies directed against CD45, CD4, CD8, CD14 and CD19 (all from Becton Dickinson, San Jose, CA, USA) at room temperature (RT) for 15 min. The cells were washed twice with phosphate buffered saline (PBS) and further incubated for 15 min at RT with Streptavidin- Cy5 (Becton Dickenson). After additional washing of the cells three times with PBS, the pellet was resuspended in 80 µL PBS. The water soluble cross-linker Bis(sulfosuccinimidyl) suberate (BS3; Pierce Biotechnology, Rockford, IL, USA) was used to cross-link the antibodies to the cell surface following the protocol published previously.[25] About 80 µL of BS3-citrate-buffer (1.14 mg BS3/1 000 µL 5 mM citrate buffer) were added to the cells and incubated on ice for 30 min. After adding 1 mL stop reagent (10% TRIS buffer), the cells were further incubated on ice for 20 min. The tubes were centrifuged and the supernatant was removed.

The telomere staining was performed according to previously published protocols.[22,26] Briefly, the cells were resuspended in 300 µL of a hybridization mixture containing 20 mM TRIS, pH 7.1, 20 mM Nacl, 1% BSA, 75% deionized and purified formamide and either 0.3 µg/mL PNA- fluoresceine isocynate (FITC) $(C3TA2)_3$ (Invitrogen, Carlsbad, USA) or an

equivalent amount of distilled water. The tubes were incubated in a water bath at 87°C for 15 min, followed by 2 h of hybridization at RT in the dark. Four wash steps were then performed with 1 mL of a wash buffer containing 75% formamide, 10 mM TRIS, pH 7.1, 1% BSA, 1% Tween20. After removing the supernatant, 1 mL of a glucose 5% solution containing 1% BSA, 1% Tween20, 10 mM HEPES was added and the cells were centrifuged (10 min, 900 g). The final DNA counterstaining was performed by adding 300 μL PBS containing 0.06 μL/mL propidium iodide (PI) and 0.1% BSA to the remaining supernatant of 50 μL. The tubes were stored at 4°C and analysed within the next 12 h.

Analysis of the multi-color flow-FISH

Propidium iodide was used to distinguish prefixed cow thymocytes (R2, Figure 1A) from unfixed human diploid cells (R1, Figure 1A) based on DNA staining (Figure 1E–G). Cow thymocytes were used as internal controls and in order to be able to express the results in telomere fluorescence units as demonstrated previously.[27]

Human diploid cells (R1, Figure 1A) were divided into lymphocyte (R3, Figure 1B) and granulocyte (R4, Figure 1B) subpopulations based on size and granularity. Cy5-labeled antibody staining allowed to further discriminate into positive (R5 and R7, Figures 1C and D) and negative (R6 and R8, Figure 1C and D) subpopulations. The corresponding telomere length of the positive subpopulation is shown in e and f (Figure 1). The dark grey peaks represent the FITC-labeled telomere staining, the light grey peaks reflect the autofluorescence of unstained samples (Figures 1E–G).

Statistics

Telomere length was expressed as mean ± SD and indicated in TFU (1 TFU = 1 kb). If not stated otherwise, all values are based on two independent measurements. Two-sample 2-sided-*t*-tests were used to compare mean values and mean age. To analyse the clinical data, Spearman correlation coefficient and multiple linear regression was used. Significance was accepted for *P*-values <0.05.

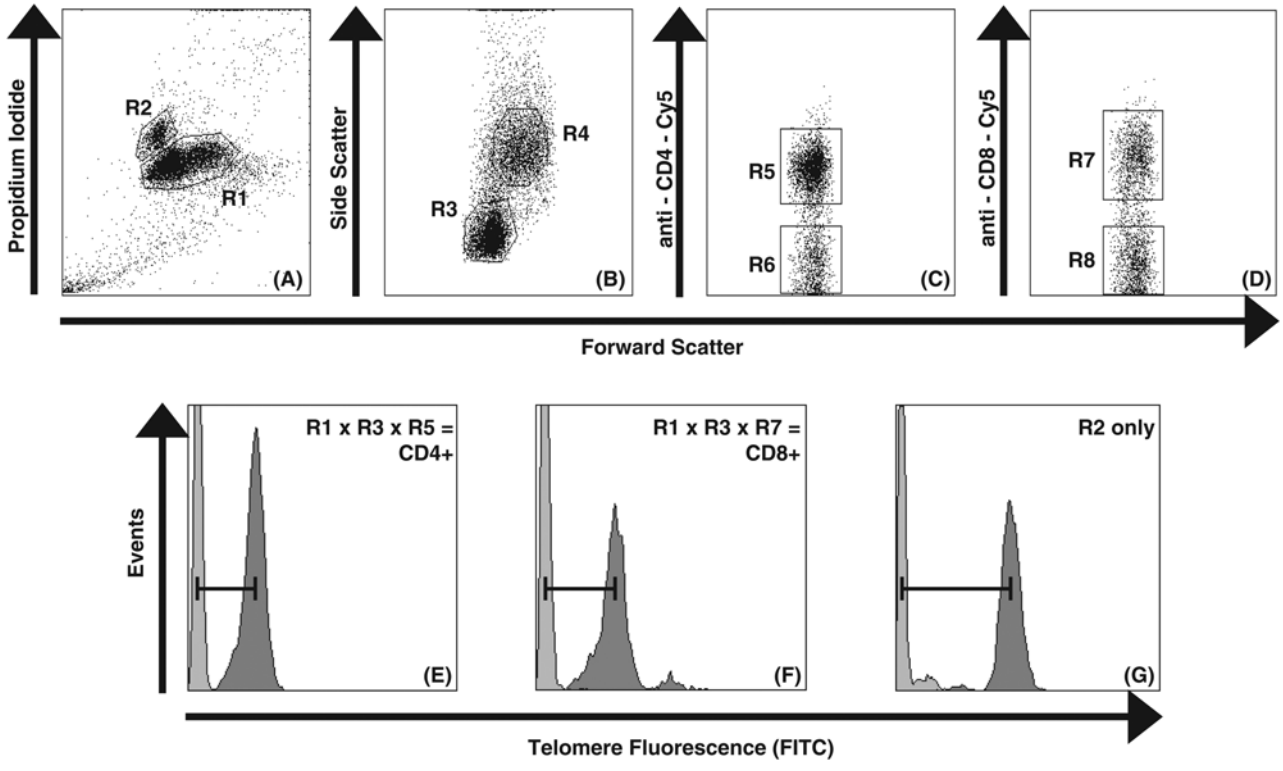

Figure 1 FACS – Scattergram of the multi-color flow-FISH. Blood sample of a 23-year-old female patient with SLE. In (A) diploid mononuclear blood cells are gated in R1 and could be easily differentiated from cow thymocytes used as internal control (in R2, G) using forward scatter and PI staining. The gated cells in R1 can be further distinguished based on size and granularity (R3 and R4 shown in (B). Cells in R3 are further separated (shown in C and D) based on their staining with the Cy5-labeled antibody. Telomere length analysis of positive (CD4+ in R5, and CD8+ in R7) and negative cells (R6 and R8, respectively) is shown in (E) and (F). The dark grey peak represents telomere fluorescence while the light grey peak reflect background autofluorescence (difference indicated by the horizontal bars).

Results

We analysed the CD4, CD8, CD14 and CD19 positive leukocyte subpopulation in 20 HD and 22 patients with SLE. To compensate for age-dependent variation in telomere length, the age of the HD and SLE patients was age-matched by group. Owing to the variability in size of an individual lymphocyte subpopulation, in some cases, the cell count was too small to determine the telomere length. For this reason, the number of individual leukocyte subpopulations analysed was different from the total number of samples included. However, this did not impact significantly on the age-adaptation.

First, we compared the four leukocyte subpopulations of SLE patients to the respective populations derived from healthy donor controls. We found no significant difference in telomere length between the CD4+ subpopulation of SLE patients (6.8 ± 1.5 TFU, $n = 22$) and HD (6.9 ± 1.1 TFU, $n = 19$, $P = 0.75$) nor in the CD8+ (7.0 ± 2.0 TFU, $n = 21$ versus 6.9 ± 1.2 TFU, $n = 19$, $P = 0.87$) lymphocyte subpopulation (Figure 2). Although telomere length in CD19+ cells from patients with SLE seem to be shorter (7.1 ± 1.0 TFU, $n = 15$) as compared with controls (7.7 ± 1.0 TFU, $n = 14$), this difference did not reach statistical significance, potentially because of the limited sample size ($P = 0.12$). Furthermore, no significant difference in telomere length in CD14+ monocytes from SLE patients (7.6 ± 1.6 TFU, $n = 21$) as compared with HD (8.0 ± 1.0 TFU, $n = 20$, $P = 0.36$) was observed.

In order to correct for intra-individual genetic variability in telomere length and based on the finding that telomere length in monocytes from SLE patients and HD did not differ significantly, we determined the difference of the telomere length between CD14 and CD4 (Δtel CD4–CD14), CD8 (Δtel CD8–CD14) or CD19 positive cells (Δtel CD19–CD14), respectively in each SLE patient and HD (Figure 3). In this intra-individual comparison we found that CD4, CD8 and CD19 positive cells, respectively had shorter telomeres then CD14 positive cells in both SLE patients and HD (all P-values <0.05). This finding confirms previous data,[28] showing that both telomere length and rate of telomere shortening in lymphocytes are typically different from myeloid cells (i.e., monocytes and granulocytes). When comparing the relative subpopulation of HD to the SLE patients however, again no significant difference in telomere length was detected (Δtel CD4–CD14; $P = 0.52$, Δtel CD8–CD14; $P = 0.22$, Δtel CD19–CD14; $P = 0.51$).

Finally, we investigated the relationship between clinical features of the 22 SLE patients (Table 1) and the telomere length of the relative subpopulation. Therefore, the difference of the telomere length (Δtel) between CD14+ and the CD4+, CD8+ and CD19+ cells, respectively was correlated with the clinical characteristics. We found no significant correlation between the Δtel values and the clinical parameters (all P-values >0.05) in our cohort of SLE patients. To further evaluate the quantitative impact of age and the corresponding clinical features on telomere length, multiple linear regression analysis was used. In summary, we did not find an age-independent significant effect of any of the the clinical features analysed on telomere length in CD4+, CD8+ or CD19+ subpopulations.

Analysing the clinical parameters and age in relation with telomere dynamics in CD14+ cells, the absolute (and relative) lymphocyte count showed a significant negative correlation on the telomere length. Further analysis of the other clinical parameters and age did not reveal any significant influence on the telomere length of the CD14+ subpopulation.

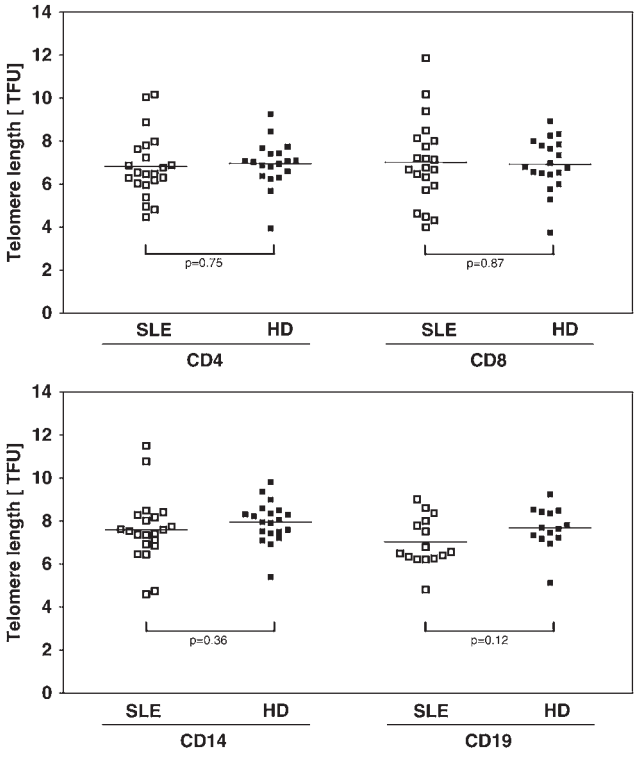

Figure 2 Scatter plot of telomere length of SLE patients and HD. Scatter plot of the subpopulations of 20 HD and 22 patients with SLE. The horizontal lines represent the mean value. All P-values are derived from a two-sample 2-sided-t-test.

Discussion

Telomeres have been implicated to be involved in replicative senescence *in vitro*[29] and *in vivo*.[30] Altered

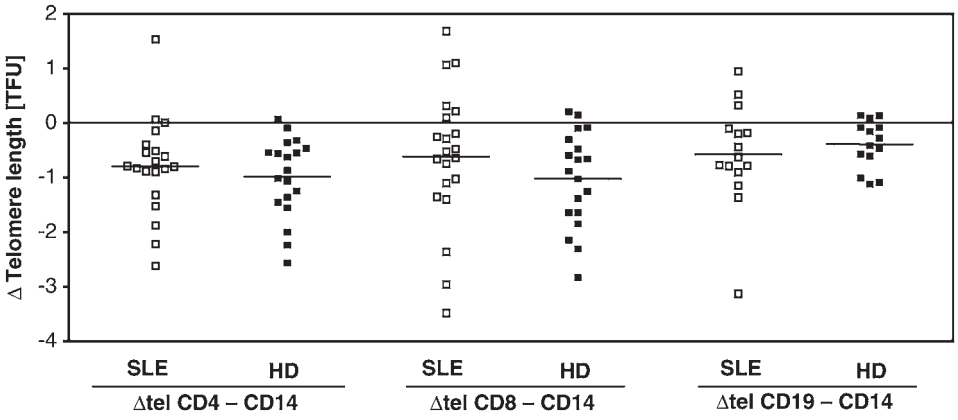

Figure 3 Difference (Δtel) between the relative lymphocyte subpoplation and monocytes. Scatter blot of the difference in Δtel between CD14+ and CD4+, CD8+ or CD19+, respectively. The horizontal lines represent the mean values. All mean values differ significantly from 0, but no difference was found in all three groups between HD and SLE patients.

telomere maintenance due to mutations in members of the telomerase complex has been shown to be linked to exhaustion of hematopoietic bone marrow function in dyskeratosis congenital.[31,32] Even in acquired disorders of the lympho-hematopoietic system, accelerated telomere shortening (e.g., reflecting increased cellular turnover) has been shown to be linked to disease stage, prognosis and response to treatment (reviewed in[5]). The aim of our study was to characterize telomere length in lymphocyte subpopulations and in monocytes of patients with SLE in comparison with healthy individuals. Monocytes have been shown to play a central role in the pathophysiology of autoimmunity in SLE. As a reflection of presumably increased cellular turnover rate (of their precursors), monocytes should be characterized by shortened telomere length. Unexpectedly, we could not detect such a difference in telomere length between patients and healthy controls. This finding could be explained by the following assumptions. First, monocytes in patients with SLE could be characterized by increased telomerase expression. This however, seems unlikely since in nonmalignant myeloid cells, telomerase activity typically becomes down-regulated with final differentiation. Secondly, since activated monocytes loose CD14-expression, for example, while they differentiate into DCs, by preselection of CD14+ cells, we could have preferentially analysed telomere length in nonactivated monocytes in the patients blood. Finally, since monocytes are thought to represent postmitotic cells with a limited life-span, stimulation and loss of these cells into other cellular compartments (leading to reduced number of CD14+ cells in the blood of patients with SLE[10] would only be reflected by telomere shortening once increased recruitment of cells from the progenitor into the differentiated monocyte compartment (requiring increased progenitor and/or stem cells turnover) takes place. In line with this hypothesis, telomere length in monocytes has been shown to nicely reflect turnover of HSC in the bone marrow of patients undergoing allogeneic stem cell transplantation.[33] Further studies will be required in order to address and clarify these alternative explanations.

In order to selectively analyse telomere length in subpopulation of white blood cells in patients with SLE, we aimed to use a novel methodology incorporating both cell surface staining with monoclonal antibodies in conjunction with the flow FISH methodology. Inter-individual heterogeneity of telomere length has been shown to be largely because of genetic[34,35] and although controversially discussed,[36] due to socio-environmental factors.[37,38] In order to correct for the mostly genetic variability in telomere length between individuals of the same age, presumably nonaffected populations of cells have previously been used to better describe disease specific changes in telomere biology in affected cells. One such example is reflected by the analysis of non-affected (i.e., Bcr-Abl-negative) T-cells as internal controls for leukemic (Bcr-Abl-positive) myeloid cells in patients with chronic myeloid leukemia.[39,40]

In analogy to this approach and based on the results of our direct comparison between SLE patients and HD mentioned above, we therefore utilized CD14+ monocytes as internal reference in order to be able to describe more accurately telomere length dynamics in the respective lymphocyte subpopulations. Using this approach, we found indeed that CD4, CD8 and CD19 positive cells have significantly shorter telomeres than their CD14 positive counterparts in both SLE patients and HD (all P-values <0.05).

Table 1 Clinical features of the 22 patients with SLE

Number	*Age (years)*	*Duration (months)*	*Leucocytes (n/μL)*	*Lymphocytes (in%)*	*Lympocyte (n/μL)*	*Hemoglobin (g/dL)*	*Platelets (10 3/μL)*	*ESR (mm/1st h)*	*Neopterin (ng/mL)*	*CRP (mg/mL)*	*C3 (mg/dL)*	*C4 (mg/dL)*	*Treatment*	*SLEDAI*
1	54.0	83	10450	11.4	1190	14.0	310	7	1.6	0.08	84	23	PRED,AZA	4
2	27.6	129	8530	13.0	1110	8.2	355	32	2.0	0.02	73	13	PRED, CSA	2
3	39.2	28	4940	27.3	1350	10.7	230	30	3.7	0.08	112	27	PRED, CSA	2
4	27.6	63	8050	19.9	1600	11.7	538	8	n.d.	0.75	71	12	PRED	4
5	45.6	104	6900	9.8	680	14.9	275	5	1.5	0.02	n.d.	n.d.	PRED, AZA, HCQ	2
6	26.7	65	5120	14.2	730	14.2	228	11	3.1	0.05	131	33	AZA	0
7	21.5	22	7820	8.4	660	12.8	408	n.d.	0.9	0.01	94	16	PRED, AZA, HCQ	3
8	50.8	158	1440	47.1	680	14.9	118	14	2.0	2.21	94	14	PRED, AZA	4
9	39.9	62	6760	19.7	1330	12.4	319	21	n.d.	0.17	n.d.	n.d.	PRED	10
10	59.6	112	5970	5.4	320	14.3	190	19	2.4	0.59	96	17	PRED, AZA	0
11	65.2	95	7760	12.0	930	13.1	320	25	n.d.	0.22	n.d.	n.d.	PRED	0
12	75.4	194	9280	17.2	1600	14.0	228	7	3.4	0.34	103	21	PRED	0
13	38.4	156	4800	1.0	50	12.8	207	17	n.d.	0.10	59	12	PRED, AZA, IG	12
14	41.9	261	5990	7.9	470	12.9	182	23	4.1	0.06	73	16	PRED, MYCO	2
15	31.8	136	6040	16.7	1010	11.0	183	n.d.	n.d.	0.19	n.d.	n.d.	PRED, CYC	0
16	23.3	110	7420	5.8	430	8.9	165	12	n.d.	1.20	n.d.	n.d.	PRED, AZA	0
17	30.2	150	10690	15.6	1670	15.4	328	11	3.3	0.49	111	21	PRED, AZA	2
18	55.6	17	8260	23.0	1900	15.1	229	4	3.1	0.12	77	11	PRED, HCQ	4
19	67.3	226	9190	32.5	2990	14.2	188	8	n.d.	0.51	142	21	PRED, AZA, NSAID	0
20	37.7	261	7970	29.9	2380	16.8	337	2	1.9	0.13	123	17	PRED,HCQ	4
21	46.5	109	6730	23.8	1600	16.6	119	6	2.3	0.11	82	14	PRED, DAN	6
22	50.8	21	12530	2.3	290	14.0	369	30	6.1	4.47	110	18	PRED, AZA, HCQ	2

PRED, prednisolon; AZA, azathioprine; IG, immunglobuline; MYCO, mycophenolatmofetile; CYC, cyclophosphamide; HCQ, hydroxychloroquine; NSAID, nonsteroidal anti-inflammatory drug; DAN, danazol; n.d., not done.

Using conventional flow-FISH, Fritsch *et al.* found that telomeres are shortened when CD4+ cells of SLE patients differentiate from naive to terminally differentiated T-cells.[15] The comparison to their previously reported data[41] do not reveal any difference between the telomere length in the corresponding differentiation steps of CD4+ cells in HD and SLE patients. Although a direct comparison of the data is not possible, these results are in line with our data presented here.

Further, Klapper *et al.* compared CD4 and CD8 positive cells but did not find a significant difference in telomere length between SLE patients and HD.[17] Using magnetic bead-coupled antibodies for enrichment of phenotypically defined cell populations followed by the TRF-assay to determine the telomere length, only a small group of patients were investigated. We reproduced the results in a larger group of age-matched patients and HD by means of multicolor flow-FISH. We were able to confirm the findings in the direct comparison of HD to SLE patients as well as in the intra-individual comparison using CD14+ internal control cells and could extend it to the CD19+ B-cell compartment. The latter results were compared with data obtained by Kurosaka *et al.* who again used magnetic bead-coupled antibodies to CD19 and CD3, respectively for enrichment followed by conventional flow-FISH in a sample size comparable with our study.[19]Again, in line with our observations by multicolor flow-FISH, Kurosaka *et al.* did not find any difference between telomere length in CD19+ cells from HD and SLE patients.

As reported previously, clinical parameters may have an effect on telomere length in lymphocytes.[20] We therefore tried to reduce the influence of age on the telomere length using two different approaches. On the one hand we used monocytes as an internal control, on the other hand multiple linear regression was used to evaluate the effect of clinical parameters *per se* on telomere length. In line with the results of Kurosaka *et al.*[16] and Honda *et al.*[18] we found no significant correlation between disease activity and the telomere length in the lymphocyte subpopulations studied. Given the low mean SLEDAI index in our patient cohort, it might be speculated that as opposed to the borderline effect of the SLEDAI index on the telomere length in total MNC reported by Wu *et al.*[20] we might not have been able to pick up such a difference in our study on phenotypically-defined subsets. Concerning the other clinical parameters, especially disease duration, grade of lymphopenia and treatment, we did not find any significant effect on the lymphocytes telomere length. Again, our data is in accordance to previously reported results,[18,20] although Wu *et al.* reported a possible effect of the immunosuppressive treatment on the telomere length. As the majority of our patients have no significant laboratory abnormalities (except lymphopenia), these findings might also be explained due to the low mean disease activity of the cohort (Table 1).

By investigating the relation of the CD14+ cells to clinical parameters, only the lymphocyte count was found to have a significant effect on telomere length. Surprisingly, patients with normal lymphocyte counts seemed to have shorter telomeres compared to patients with lymphopenia. Based on the small sample size of the study, the lack of any correlation between clinical data and lymphocyte telomere length and the overall low disease activity of the cohort, the results should be interpreted with caution. In addition, the interpretation of the causes of lymphopenia (disease- versus treatment-related) in this patient population is restricted. Therefore, the relation of telomere length in monocytes to lymphocyte count should be clarified in larger follow-up study.

In summary, telomere length has gained increasing interest as a surrogate marker for both the proliferative history and replicative reserve of normal somatic cells. In our study, which utilized a novel methodological approach, we were able to confirm previous findings on telomere length in lymphocyte subpopulations of patients with SLE and to extend the studies to the monocyte compartment. The use of the multicolor flow-FISH allows the analysis of defined cell subpopulations in clinical scale sample sizes and might help to gain a deeper insight into the pathogenesis and disease progression of both immunological as well as hemato-oncological diseases (reviewed in[5]).

Acknowledgements

This work was supported by a grant of the 'Deutsche Forschungsgemeinschaft (SFB510, Project A6)' as well as the 'Eppendorfer Krebs- und Leukämiehilfe'. Further we like to thank Iris Schäfer and Bettina Kirchner for their excellent technical assistance.

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