Telomere length and oxidative stress in the progression of Alzheimer's disease

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Abstract

Telomere length has been widely used as a marker of biological aging due to its sensitivity to the environment along with its relation to cellular division. Alzheimer's disease (AD) is the most common cause of dementia and aging is its major risk factor. Increased cases of dementia imply a first response to consider in view of an aging population; hence, the study of this phenomenon has become a global priority.

Our aim was to explore telomere length (TL) as a molecular change caused by the progression of AD in a transgenic murine model. TL was assessed by qPCR in both brain tissue (hippocampus) and peripheral blood and compared between three age groups: 5, 9, and 13 months. In addition, a possible effect of oxidative stress on TL and AD progression was explored.

Decreased TL due to AD progression seems to be due to an increased oxidative stress proper of the disease although we found no association between the two, which can be due to the impaired neurogenesis in the hippocampus in AD. TL and oxidative stress changes in peripheral blood cells of this mouse model suggest that AD affects the individual systemically, and may be suitable for developing diagnostic strategies.

Keywords: lifespan, neurogenesis, hippocampus

Background

Alzheimer's disease (AD), the most common cause of dementia, is defined by progressive and irreversible neurodegeneration of the central nervous system that eventually leads to the gradual decline of cognitive function [1], 2]. The main feature of this disease is the synaptic deregulation that begins in the hippocampus and eventually leads to the loss of neurons [3], mainly due to structural changes that involve the accumulation of neuritic plaques of the β-amyloid fibrillary peptide and neurofibrillary tangles of hyperphosphorylated tau protein [4]. In addition to these neuronal lesions, some biomarkers of genomic instability have been found in patients with AD including micronuclei (markers of loss and breakage of chromosomes), aneuploidies of chromosome 21, shortening of telomeres in lymphocytes and fibroblasts [5], and increased oxidative stress markers [6].

Telomeres are essential non-coding DNA complexes associated with double and single strand DNA found at the ends of linear chromosomes to protect them from fusion and degradation [7]. Telomeres are sequences of 6 base pairs repeated in tandem; their length varies according to the species and chromosome. Each time a cell divides, its telomeres are progressively shortened; the increase of cell proliferation in a tissue results in the progressive shortening of telomeres, which triggers a DNA damage response, if repaired, the proliferation is restored. On the contrary, if the deterioration is irreversible, the cell keeps the cell cycle at halt and can be directed to apoptosis or cellular senescence [8], [9]. Telomere length depends on several factors such as the speed of degradation of the telomeres, and the speed and time of action of telomerase in each chromosome [10,11].

Hence, telomere length decreases differentially with time in all mitotic tissues.

On that matter, the brain of patients suffering AD present a significant extent of oxidative damage associated with the abnormal marked accumulation of β-amyloid and the deposition of neurofibrillary tangles [12]; stress contributes to a significant decrease in telomerase activity and consequently causes an increase in the telomere-shortening rate [13]. Biochemical studies show TTAGGG repeats are preferred sites for iron binding and iron mediated Fenton reactions, which generate hydroxyl radicals that induce the 5' cleavage of GGG [14]. There is a minimal shortening of less than 20 bp per cell division in cells with high antioxidative capacity, rates increase in cells with lower antioxidative defence. Cultivating cells under enhanced oxidative stress like mild hyperoxia (40% normobaric oxygen) shortens the telomeres prematurely and reduces the replicative lifespan accordingly [15]. Similarly, telomere length has been related to lifespan [16]; during aging 50-150bp of telomeric DNA is lost with each proliferation cycle [17].

Specifically, it has been reported that patients with AD show shorter telomeres in peripheral blood mononuclear cells, monocytes, T and B cells [18], buccal cells, and leukocytes compared to controls [5]. Also, significant differences in leukocyte telomere length (LTL) between controls, amnestic mild cognitive impairment, and AD patients has been recently reported; the authors found a positive association between LTL and cognitive performance measured by the Mini Mental State Examination Score and propose that the shortened LTL observed in AD patients stems from progressive telomere

erosion possibly correlated with the cognitive decline characterizing conversion from amnestic mild cognitive impairment to AD [19].

Telomere length has been associated with life span since it reflects the number of times a cell has divided [16], as well as disease conditions as mental stress, obesity, smoking, type 2 diabetes mellitus, ischemic heart diseases, AD and Parkinson's disease [20–23] Hence, we decided to evaluate changes in telomere length and oxidative stress due to the progression of AD in a murine model. Since our model has a genetic disposition to develop AD we expected to see an intrinsic effect of disease progression on telomere length.

Methods

Animals

Homozygous 3xTg-AD (3xTg-AD; B6; 129-Psen1 <tm1Mpm> Tg (APPSwe, tauP301L) 1Lfa) male mice were used as the AD murine model, and B6129SF2/J WT male mice were used as controls. Mice were purchased from Jackson Laboratories. All procedures were performed in accordance with the current rulings in Mexican law (NOM-062-ZOO-1999) and with the approval of IMSS Science and Ethics Committees (COMBIOETICA09CE101520130424) with number R-2012-785-049. Mice were housed in a 12:12 light/dark cycle at 20-22ºC with water and food *ad libitum*. All animals were genotyped. Three ages were used for this project 5, 9, and 13 months. Groups were as follows: 5mo 3xTgAD n=7, 5mo WT n=12; 9mo 3xTgAD n=9, 9mo WT n=12; 13mo 3xTgAD n=8, 13mo WT n=12.

Tissue samples and DNA extraction

Blood samples were obtained from the mandibular vein of all mice (~0.2ml). The blood sample was first treated with erythrocyte lysis buffer. Brain hippocampus was dissected and tissue was homogenized with lysis buffer. DNA was extracted according to the manufacturer's instructions (Thermo Scientific DNA extraction kit, K0512). Purified DNA samples were stored at -70 °C until use.

Telomere length assessment

We followed the qPCR (StepOnePlus Real-Time PCR System, Applied Biosystems) method published by O´Callaghan and Fenech [24]. An absolute quantification method by introducing an oligomer standard and a standard curve was used to determine telomere length; the 36B4 housekeeping gene was used as an endogenous calibrator. The number of copies of telomeric repeats was determined by the standard curve of telomere standard, while the standard curve of 36B4 STD was used as control. Results are expressed as absolute telomere length (aTL).

To determine telomere length the Ct values of each sample were extrapolated in their corresponding curves by a linear regression test. The Maxima SYBR Green/ROX qPCR Master Mix 2X (ThermoScientific, California, USA) was used. The cycling conditions for both genes were: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, followed by a melting curve.

Oxidative Stress measurements

All measurements were performed on samples from 5 and 9 month-old mice from both strains.

Reactive oxygen species (ROS)

Measured by the oxidation of 2-7-dichlorofluorescein (DCFH) to the fluorescent oxidized compound 2-7- dichlorofluorescein (DCF) by the presence of hydrogen peroxide. DCFH can be oxidized by several reactive intermediates, so it cannot be used to determine the presence of a specific reactive species.

To evaluate the formation of ROS by fluorometry, homogenates from the dissected brain regions and blood plasma were used, 60µl of protein from each sample was used for a final volume of 200µl with 1x PBS buffer, 10µL of a DCFDH-diacetate at 75 µM, samples were incubated in the dark for 30 min at 37ºC, then centrifuged at 10,000 rpm for 10 min; the supernatants were read in a fluorometer at an excitation wavelength of 480nm and emission of 532. Results are expressed in nM of DCFH in mg of tissue samples or nM of DCFH in µL of serum.

Lipid peroxidation

Measured by the production of malondialdehyde (MDA); 120µL of the homogenate of the different dissected brain regions or the plasma were mixed with 60µL of 1x PBS and 120µL of the TBA reagent (0.375g of TBA + 15g of trichloroacetic acid + 2.54ml of concentrated HCl). The samples were placed in a boiling bath (94 °C) for 20 min. Subsequently, the samples were centrifuged at 10,500 rpm for 15 min and the optical density of the

supernatant was determined (Biotek plate reader) at a wavelength of 532 nm. Results are expressed as µM of MDA in µL of serum and µM of MDA in mg of tissue sample.

Mitochondrial functionality

Measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)bromide-2,5-diphenyltetrazole (MTT nM/µL); 100µL of brain tissue homogenate was used, to which 10µL of an MTT solution (5mg / mL) was added, and incubated for 30 minutes at 37 °C in the dark. Once the incubation time had elapsed, the samples were centrifuged at 12,000 rpm for 3 minutes. The supernatant was removed and 500µL of isopropanol-acid was added to the pellet where it was suspended. Samples were read at a wavelength of 560 nm in a plate reader. Results are expressed as µM of MTT in mg of tissue.

Statistical analysis

Quantitative variables are presented as arithmetic mean and standard deviation (mean ± SD) per age group: 3, 5, and 13 months, and evaluated with one-way analysis of variance (ANOVA) on ranks.

Boxplots show the distribution of telomere length, ROS, MDA or MTT among age groups. Their p-values were calculated by Kruskal-Wallis non-parametric test followed by a post-hoc test (Dunn's test), when statistical significance between groups was obtained, and in order to discriminate the specific groups with significantly different medians from the others.

Results

To explore molecular changes due to the progression of Alzheimer's disease in a transgenic murine model (3xTgAD), we studied telomere length as well as the possible effect of oxidative stress on this phenomenon. Table 1 shows the mean values of telomere length, measured in whole blood cells and hippocampus at 5, 9, and 13 months, as well as ROS, lipid peroxidation and MTT at 5 and 9 months, for both transgenic and wild type strains.

Table 1.

Mouse	Months	Tissue	Mean ± SD			
			Telomere	ROS	MTT	TBA
Wt	5	Estriado	6094.97 ± 1538.87	144.08 ± 78.99	0.046 ± 0.007	0.616 ± 0.368
		Hypocampus	172072.90 ± 132854.48	100.79 ± 67.62	0.077 ± 0.029	0.503 ± 0.165
		Plasma	131346.33 ± 22679.50	635.66 ± 158.87	----------	0.068 ± 0.05
	9	Estriado	560398.07 ± 133436.62	201.26 ± 40.85	0.036 ± 0.025	0.548 ± 0.127
		Hypocampus	78784.62 ± 28704.86	215.2 ± 40.28	0.029 ± 0.005	0.941 ± 0.281
		Plasma	133213.27 ± 124150.87	1295.01 ± 252.54	----------	0.094 ± 0.059
	13	Estriado	20652.22 ± 17669.37	----------	----------	----------
		Hypocampus	134037.59 ± 85636.12	----------	----------	----------
		Plasma	96708.28 ± 60475.63	----------	----------	----------
Tg	5	Estriado	7805.58 ± 2798.92	189.07 ± 29.9	0.069 ± 0.019	0.386 ± 0.064
		Hypocampus	106920.66 ± 64403.23	444.51 ± 197.26	0.104 ± 0.013	0.346 ± 0.137
		Plasma	149004.25 ± 20878.8	1777.15 ± 339.49	----------	0.095 ± 0.037
	9	Estriado	677934.86 ± 213583	157.75 ± 27.53	0.036 ± 0.009	0.99 ± 0.075
		Hypocampus	104829.49 ± 62346.53	1442.3 ± 1395.46	0.029 ± 0.013	1.245 ± 0.384
		Plasma	153357.85 ± 64667.27	3796.08 ± 1061.87	----------	2.805 ± 1.488
	13	Estriado	37501.01 ± 19308.19	----------	----------	----------
		Hypocamp	52223.5 ± 49501.22	----------	----------	----------
		Plasma	15442.77 ± 9297.83	----------	----------	----------

Telomere length

Telomere length in whole blood cells of the transgenic strain showed significant differences between age groups ($p<0.01$), the 13 month old group showed differences between 5 and 9 month old groups ($p<0.05$). No significant differences were found between age groups form the wild type strain ($p=0.70$). When compared between mice strains, significant differences were found between 13-month-old transgenic and wild type mice ($p<0.01$) as can be seen in Figure 1a.

Telomere length was also measured in the hippocampus, one of the first structures affected by AD. We found no significant differences between age groups within strains ($p=0.29$ for transgenics and $p=0.36$ for wild type) or between wild type and transgenic mice ($p=0.29$) as can be seen in Figure 1b.

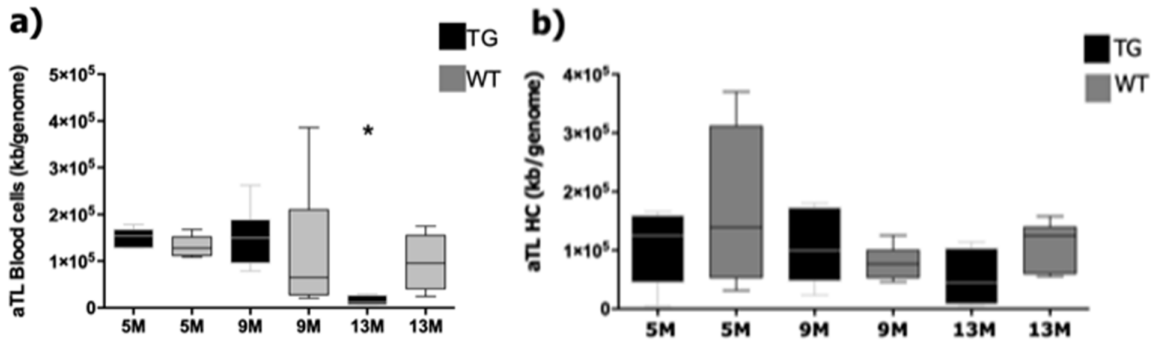

Figure 1. Absolute telomere length measured in transgenic (black) and wild type (grey) mice of 5, 9 and 13 months of age. a) Blood cells telomere length decreases with age and is significant between 5 and 13 and 9 and 13 months of age in transgenic mice, but no significant changes were found for wild type. Significant differences between strains of 13 months of age were found. b) Hippocampus telomere length is not affected by strain or age group.
aTL, absolute telomere length; TG, transgenic strain; WT, wild-type strain; M, months
* Statistically significant difference (p-value cut-off $p<0.05$) obtained by Kruskal-Wallis.

Oxidative stress

In order to determine whether oxidative stress was involved in the observed telomere length reduction, we evaluated ROS (measured by DCFH) and lipid peroxidation (measured by MDA) at 5 and 9 months of age. In blood cells, both ROS and lipid peroxidation showed a significant increase between age groups within strains. As shown in Figure 2a, ROS in blood cells of 9 months old transgenic mice are significantly higher than that of 5 months old mice (p=0.0090). ROS also increased between 5 and 9 months old groups of the wild type strain (p=0.0039). ROS increases with age in both strains, but significantly more in the transgenic mice where the 9 months group also showed differences between strains (p=0.0062).

On the other hand, Figure 2b shows lipid peroxidation, measured by MDA (µM/µL), increases only in the 9 months old transgenic mice group, which is significantly different from every other group (p=0.0201).

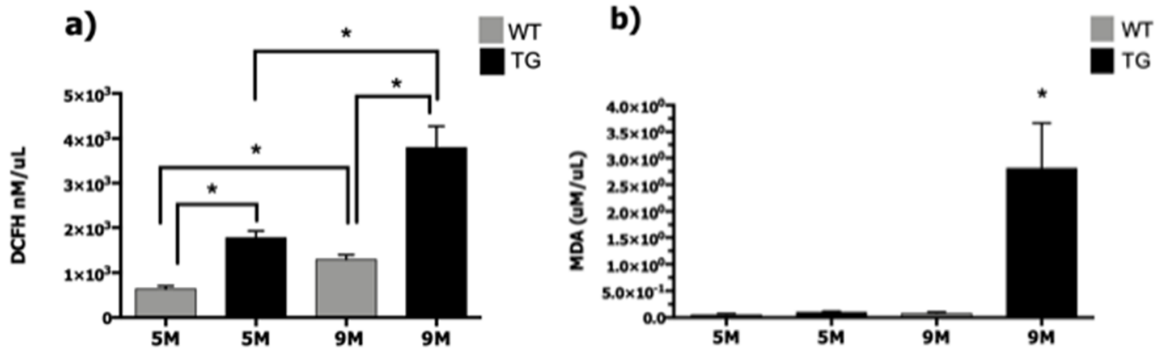

Figure 2. Oxidative stress in blood cells of transgenic (black) and wild type (grey) mice of 5 and 9 months of age. a) Measurement of ROS by DCFH. Blood cells ROS increases with age in both strains and is statistically significant at 9 months of age between strains. b) Lipid peroxidation measured by MDA. Significant differences were found between 9 month old transgenic mice and every other group.
DCFH, 2-7-dichlorofluorescein; MDA, malondialdehyde; TG, transgenic strain; WT, wild-type strain; M, months
* Statistically significant difference (p-value cut-off p<0.05) obtained by Kruskal-Wallis.

When measured in the hippocampus, ROS showed an increase with age within the strains (p=0.0062; p=0.0105) and differences in both ages between wild type and transgenic mice (p=0.0250; p=0.0500) as can be seen in Figure 3a. Lipid peroxidation showed a significant increase between 5 and 9 months old mice for both strains (p=0.0090; p=0.0163) and no differences between strains (p>0.05) (Figure 3b). Similarly, mitochondrial viability (measured by MTT) decreased with age in both groups (p=0.0039; p=0.0090) and showed no differences between strains (p> 0.05) as shown in Figure 3c.

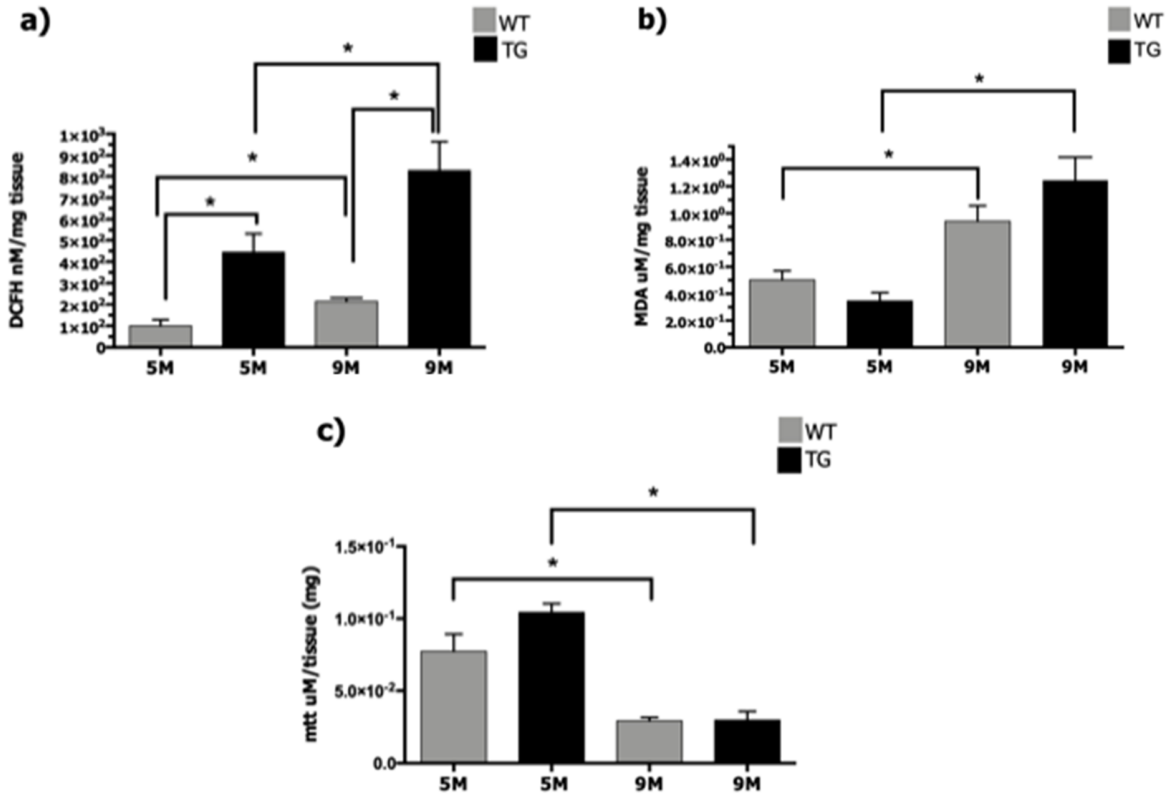

Figure 3. Oxidative stress in hippocampus homogenates of transgenic (black) and wild type (grey) mice of 5 and 9 months of age. a) Measurement of ROS by DCFH. ROS increases with age in both strains and is statistically significant at 5 and 9 months of age between strains. b) Lipid peroxidation measured by MDA. Significant differences were found between 5 and 9-month groups of both strains but not between strains. c) Mitochondrial viability measured by MTT. Significant differences were found between 5 and 9-month groups of both strains but not between strains

DCFH, 2-7-dichlorofluorescein; MDA, malondialdehyde; mtt, 3-(4,5-dimethylthiazol-2-yl)bromide-2,5-diphenyltetrazole; TG, transgenic strain; WT, wild-type strain; M, months

* Statistically significant difference (p-value cut-off p<0.05) obtained by Kruskal-Wallis.

When analysing the data, no correlation was found between the blood cells increase of ROS or lipid peroxidation and telomere length shortage for 5 or 9 months-old transgenic and wild type mice (Table 2).

Table 2. Correlation between telomere length and oxidative stress.

				Correlation (Rho Spearman)		
Mouse	Months	Tissue		ROS/telomere	MTT/telomere	TBA/telomere
Wt	5	Estriado		**0.88 (p=0.019)**	0.40 (p=0.6)	0.54 (p=0.266)
			N	6//6	4/6	6/6
		Hypocampus		0.31 (p=0.544)	-0.48 (p=0.329)	0.25 (p=0.623)
			N	6/6	6/6	6/6
		Plasma		0.48 (p=0.329)	ND	0.70 (p=0.188)
			N	6/6		5/6
	9	Estriado		0.14 (p=0.787)	0.029	0.77 (p=0.072)
			N	6/7	(p=0.957)	6/7
					6/7	
		Hypocampus		0.2 (p=0.704)	-0.48 (p=0.329)	-0.14
			N	6/6	6/6	(p=0.787)
						6/6
		Plasma		0.42 (p=0.397)	ND	**0.90**
			N	6/9		**(p=0.037)**
						5/9
Tg	5	Estriado		0.70 (p=0.188)	0.60 (p=0.40)	0.80 (p=0.20)
				5/6	4/6	4/6
		Hypocampus		-0.20 (p= 0.747)	-0.10 (p=0.873)	-0.30
				5/5	5/5	(p=0.624)
						5/5
		Plasma		0.30 (p=0.624)	ND	-0.80 (p=0.2)
				5/5		4/5
	9	Estriado		**-1.00 (p=0.01)**	0.40 (p=0.60)	-0.30
				3/7	4/7	(p=0.624)
						5/7
		Hypocampus		**0.90 (p=0.037)**	0.70 (p=0.188)	0.00 (p=1.00)
				5/6	5/6	5/6
		Plasma		0.7 (p=0.188)	ND	0.50 (p=0.667)
				5/11		3/11

Discussion

Telomere length and oxidative stress are independently affected by AD progression in the 3xTg-AD murine model. Shorter telomeres were found in blood cells of older transgenic mice compared to younger and wild type mice. This could be a reflection of a systemic effect due to the progression of AD in our murine model since it is not aging but the disease itself that is causing the changes in telomere length.

In accordance with our findings, two different studies have reported shorter telomere length in blood cells and leukocytes of patients with AD compared to controls [5,18]. What we have now found is that it is the progression of the disease that favours telomere shortage in blood cells. Also, a previous paper reported that 8OHdG [25], a DNA oxidative stress marker, is elevated in lymphocytes of AD patients compared to a control group. Accordingly, we found increased oxidative stress, measured by ROS and lipid peroxidation, in blood cells of older compared to younger transgenic mice.

On the matter, an association between elevated oxidative stress and telomere length has been documented [13]. Nonetheless, no association was found between the increase in oxidative stress and telomere length in our model. For that purpose, 8OHdG would have been a better biomarker to associate with telomere length since it is directly related to DNA damage due to oxidative stress. Unfortunately, this measurement was impossible to obtain at the time.

Regarding our findings of telomere length in the hippocampus (HC), we only found a tendency, but no significant differences between the transgenic 13 months old mice and

the younger groups. It seems as if the progression of AD would eventually produce changes in telomere length but we were unable to observe it. Changes in telomere length in brain tissue are tricky since most neurons do not replicate and specifically the hippocampus shows reduced neurogenesis in AD [26]. Therefore, shortening is either due to environmental factors or to an increased replication of glial cells [5], a distinction we did not make at the moment.

Similarly, Franco and co-workers [27] found no changes in telomere length of the subcortical and granular areas as well as the hippocampus between transgenic mice for APP and their controls. They concluded that the accumulation of amyloid beta has no relation to telomere shortening. Moreover, one study found no changes in telomere length in the cerebellum of patients with AD against controls [28]. Hence, progression of the disease does not seem to affect telomere length in brain tissue as it does on blood cells.

Regarding aging and telomere shortening, a positive correlation between age and telomere length [28] has been reported in human tissue and peripheral blood cells, a feature we did not find in any of our mice, wild type or transgenic. Accordingly, a comparative study in mice established no correlation between telomere length and age in these animals; the replicative aging mechanism controlling the number of cell divisions where telomere length is shortened, as has been reported in humans, appears to be different in mice [29].

Taking it all into account, human and mice studies have both described no changes in telomere length in brain tissue samples but a shortening in telomere in whole blood cells

of AD patients. Our results show that a shorter telomere is due to the progression of the disease supporting the newest discovery of AD being a disease that does not only affect the central nervous system but has a systemic effect. Since telomere length is believed to reflect environmental effects rather than cell division, specifically in mice [30], the increase in oxidative stress could be the reason for the DNA damage we observed in blood cells.

Aging as well as AD has an effect on oxidative stress [31,32], which we were able to confirm here. Both mice strains had increased markers of oxidative stress for blood and tissue samples when compared to a younger group, but transgenic mice showed even higher values suggesting an additive effect of age and Alzheimer's disease.

Conclusion

Oxidative stress and telomere shortening in peripheral blood was correlated with the progression of the disease but not with each other. Telomere shortening due to Alzheimer's progression seems to be due to an increased oxidative stress proper of the disease. Lack of association in telomere length in the hippocampus could be due to the lack of divisions of neurons. The presence of structural or functional changes in peripheral blood cells of this mouse model suggests that AD affects the individual systemically due to its progression.

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