Senescence and longevity in turtles: What telomeres tell us

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Abstract. Turtles exhibit approximately the same longevity as humans. However, senescence, which is a major characteristic of mammals ageing, has not been clearly demonstrated in these species nor in other Reptiles. We have checked if the telomere length reduction observed in humans during ageing and being one of the causes of mammalian senescence is also observed in the European freshwater turtles. No difference in telomere length is observed between genomic DNA of embryos and adults of this species.

Key words. Senescence, *Emys orbicularis*, chelonian.

Introduction

Ageing in mammals is characterized by senescence : increasing mortality and occurrence of several typical diseases (Schächter, 1993). Several factors are thought to play a role in senescence, which is probably not a mono-factorial phenomenon: oxidative stress (Sohal and Orr, 1998), mitochondrial dysfunction (Nakahara et al., 1998), thymus-based degeneration or telomere length reduction (Lundblad and Wszostak, 1989; Wright and Shay, 1992) have been all invoked to explain this phenomenon. This last factor is one of the most promising to elucidate the phenomenology of senescence being both a marker of ageing (Harley et al., 1990) and a cause of senescence (Lundblad and Wszostak, 1989). Telomeres are reduced by a purely mechanical process at each cell division when telomerase activity is absent or insufficient in cells (Wright and Shay, 1992). When a telomere becomes deleted, the chromosome becomes unstable and the gene regulation around the telomere position changes and alters the normal function of the cell (Melek and Shippen, 1996; Wright and Shay, 1992). As a consequence, the $p16^{INK4a}$/retinoblastoma pathway is activated that leads to senescence (Kiyono et al., 1998). Telomerase activity is not observed in most normal human somatic cells. This factor makes also the link between cellular and organismic senescence because telomere reduction has been shown to occur both *in vitro* and during human ageing (Harley et al., 1990).

Turtles are long-lived vertebrates, being able to live more than 100 years in captivity, and exhibiting a very high annual survival rate in natural conditions (Gibbons and Semlitch, 1982). However, the characteristics of mammalian senescence has never been clearly demonstrated in these species: no increase of mortality with ageing is observed, the reproductive capacity of females continues to grow during their life-span, and no ageing-specific diseases are known except an alteration of the caparace by mechanical abrasion on soil. The senescence is in fact very difficult to demonstrate in natural conditions. For example, higher mortality rate during ageing can be a consequence of

senescence but also a simple consequence of ageing with high level of interindividual competition.

The telomeres of all vertebrates are mainly constituted of the same 6 base repeats, (TTAGGG) (Meyne et al., 1989). *In situ* hybridizations in 12 species of two Sauropsid clades (*Aves* and *Squamata*) have demonstrated that the (TTAGGG) repeats are only shared at the telomeric position of chromosomes (Meyne et al., 1989). We have checked if a reduction in telomere length can be observed in the European freshwater turtle (*Emys orbicularis*) which exhibit approximately the same longevity as humans, but without known senescence. The mean telomere length in the genome has been compared in embryos and adults of this species.

Materials and methods

Genomic DNA has been extracted from circulating blood cells of embryos or adults from the same population (Etang neuf, Brenne, France) according to the method of Jeanpierre (1987) followed by a phenol/chloroform purification (Sambrook et al., 1989). Genomic DNA has been cut by *Alu* I enzyme (Boehringer) for 8 hours at 37°C. Samples have migrated through an agarose gel (0.8%) in TAE buffer (Sambrook et al., 1989). NaOH-denatured DNA has been transfered onto Nytran membrane using Posiblot (Stratagene). Southern-blot has been hybridized with a telomere probe $(TTAGGG)_3$ labeled with Digoxigenin (Dig-Tailing Kit, Boehringer) and revealed using chemioluminescent CSPD (Boehringer) after membrane wash using SSC 2x, SDS 0.1% at 45°C 2 times 15 min. The molecular weight marker has been revealed on the membrane using methylene blue (0.01%) in sodium acetate pH 5.2 for 10 min. To ensure the DNA was digested to completion, the same membrane has been deshybridized (NaOH 0.2M, SDS 0.1%, 2x30 min at 45°C) and hybridized again using $(GGCT)_5$ Digoxigenin-labeled primer.

Results

The ethidium bromide revelation of the *Alu* I cut DNA of *Emys orbicularis* indicates that the genomic DNA is mostly observed is very small fragments (figure 1). After hybridization with telomere probe, the bands of telomere specific sequence are very large (20 kb) and sharp (figure 2A). They do not exhibit a smear as observed in human (Harley et al., 1990). The size of bands sharing telomere sequences are not significantly different in adults and embryos ($t=0.63$, 27 DF, $p=0.53$). The membrane rehybridized with $(GGCT)_5$ probe show a normal pattern for this microsatellite that indicate the good quality of the original genomic DNA and that genomic DNA has been well cut by *Alu* I restriction enzyme (figure 2B)

Discussion

The blood cells of the European freshwater turtles do not exhibit telomere length reduction during ageing. Interestingly, the bands sharing telomere sequences appear not to form the typical smear observed in humans (Harley et al., 1990), but are rather sharp. This could indicate a control in telomere length by the expression of the telomerase enzyme in somatic stem cells in this species. Moreover, the size of this band is very large (20 kb) whereas the *Alu* I restriction enzyme is a frequent cutter enzyme in genomic DNA of *Emys orbicularis* (figure 1). This indicates that telomeres of *Emys*

orbicularis are homogeneous in sequence and very large compared to mammalian ones which are from 1 to 6 kb long (Blackburn, 1991).

These results should be considered as preliminary for two reasons. First, the age of adults used for this study cannot be determined with precision. However, they were generally older than 20 years based on number of discernable rings in scutes. Moreover, an high individual age of adults in the population is expected because the annual survivorship in this species is very high (Servan, comm. pers., see also Girondot and Pieau, 1993). The second reason to be cautious about these results is that in mammals, the telomere shortening has not been studied for red blood cells due to their lack of nucleus nor for erythrocyte stem cells. However, this last point does not alter the conclusion that telomeres are larger in *E. orbicularis* compared to mammalian ones.

Several ways can be achieved for a species to escape senescence-based telomere shortening. One solution is to possess very large telomeres so that reduction will take a very long time time before to shorten enough to activate the Rb/p16^{INK4a} pathway that leads to cellular senescence. Another solution is to possess a somatic expression of the telomerase enzyme to counteract the mechanism of telomere shortening during cell division. The preliminary results obtained in *Emys orbicularis* indicate that this species could use both solutions against telomere shortening.

This result does not prove by itself that turtle lack senescence because senescence, as defined in mammals, is a multifactorial phenomenon. For example, the Rb/p16^{INK4a} pathway can be activated also by stress (Weinberg, 1998). Moreover, lack of senescence does not mean « immortality » because the annual death probability is not null.

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Figure 1: Ethidium bromide revelation of *Alu* I cut genomic DNA of *Emys orbicularis*.

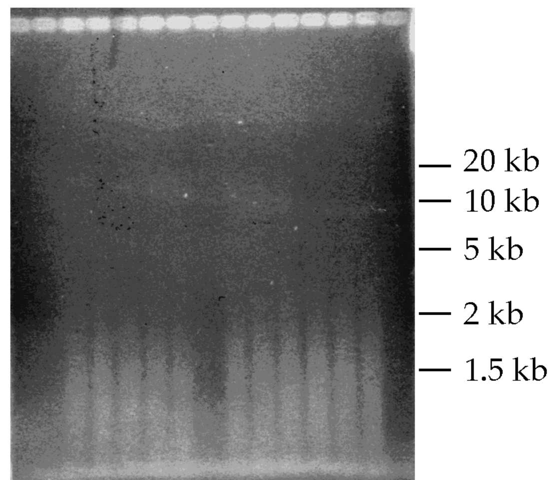

Figure 2: Southern-blot of 14 embryos and 15 adults of the European freshwater turtles' genomic DNA (unrelated animals), cut by *Alu* I and hybridized with (A) a telomere-specific probe $(TTAGGG)_3$ and (B) a microsatellite probe $(GGCT)_5$.