A FINAL REPORT OF STUDIES OF THE HAYFLICK LIMIT IN REPTILES, A TEST OF POTENTIAL IMMORTALITY

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ABSTRACT

This study was initiated to determine if it is possible that long–lived reptiles could provide a model for extension of human lifespan. Our current short lifespan is an impediment of long distance space travel and damage to senescent human tissue is also a threat for aging astronauts. This study examines mechanisms in reptiles that might benefit human longevity and tissue repair. We examined the Hayflick Limit, specifically if cultured reptilian fibroblasts underwent replicative senescence in proportion to the lifespan of the reptile from which they were obtained, as has been observed in mammals. We also tested the tissues of various reptiles for production of telomerase, an enzyme that promotes replacement of the telomere, extending the life of tissues and tissue cultures. While inadequate time has elapsed to allow most of our cultures to grow beyond the Hayflick limit, cultures from three species suggest that number of culture replications may reflect reptiles' remaining lifespan. Cultures from 20 y/o mud turtles (*Kinosternon flavescens*, lifespan 30-45years) senesced after 18-45 replications. Those from a subadult snapping turtle (*Chelydra serpentina*, possible lifespan >100 years) have multiplied more than 190 times. Cultures from an old painted turtle (*Chrysemys picta*, >35 years) senesced after 24 doublings. We found no telomerase in any tissues of these turtles other than the gonad. One of two ornate box turtle (*Terrapene ornata*) hatchlings had telomerase activity in two organs other than the gonad.

Suggestion of tissue immortality appeared with our discovery of telomerase in all of the tissues (including brain) of a teiid lizard. The potential of their cultures for replication will not be known for several months. The presence of telomerase in somatic tissues of an adult terrestrial vertebrate with no tendency for non-viral cancer, may have important human implications. Our very preliminary initial observations suggest that some reptiles may be using telomerase for extended tissue replacement or repair and that the telomerase mechanism may be used in giving some turtles longer lives than others. Replications of these experiments and additional studies are underway to confirm and explain these interesting initial, very preliminary observations.

INTRODUCTION

Two of the hazards that loom as barriers to long distance space travel are the aging of astronauts and the difficulty of repair and regeneration of injured tissue, especially in a senescent human body. Some small turtles live well beyond the age of any other vertebrate as advanced, and other reptiles retain excellent powers of tissue regeneration throughout their life. Reptiles, especially some species of turtle, are believed to grow throughout life and to have greatly postponed senescence Patnaik, (1994), Gibbons (1987). Carr (1970) demonstrated no decline in growth rate of mature breeding sea turtles (*Chelonia mydas*). In spite of this, little has been learned about the mechanisms contributing to or controlling senescence in reptiles.

Our group has successfully cultured melanomacrophages from turtles (Rund et al 1998; Johnson et al 1999) and as an offshoot of these experiments has cultured hepatocytes and fibroblasts. The rapid turnover we have observed in reptilian fibroblasts suggests that these cells may provide a tool for studying extended reptilian lifespans as was discovered by Hayflick for mammals (see review by Shay and Wright, 2000). The only such study published to date is that of Goldstein (1974) who found 100-130 population doublings for fibroblasts from young Galapagos tortoises. We will determine number of doublings of untransformed fibroblasts from young and old yellow mud turtles, *Kinosternon flavescens*, a relatively short lived turtle species and from ornate box turtles and snapping turtles, *Terrapene ornata and Chelydra serpentina,* two long lived turtle species. Fibroblasts from *Kinosternon, Chelydra, Terrapene,* and painted turtles*, Chrysemys picta,* will be examined from marked natural populations under study by Christiansen (1990) since 1973.

We will examine the clue of potentially immortal reptilian tissue provided by Simpson and Rausch (1989) suggesting that some lizard tissues reproduce without limit without malignant transformation. We are particularly interested in this because of excellent regenerative powers of some lizards. In this study we initiate measurement of telomeres in young and old cultures and in tissues from young and old reptiles to determine if telomere length is related to animal age and number of doublings. We probe for telomerase in young and old turtles to determine if some reptiles might extend their lifespan by production of this enzyme. Discovery of telomerase in large amounts in post-hatching turtles would provide the first model for this system of life extension in the terrestrial vertebrate world and would suggest that complex organisms could extend life by activating the gene for telomerase. Discovery of telomerase in adult lizard tissues could suggest importance of this enzyme in tissue regeneration. Significantly extending human lifespan and telomerase enhancement of injured senescent tissue would greatly expand our options in long distance space travel.

METHODS

Specimens and Age Estimation

Most specimens for this study were obtained at Big Sand Mound in Muscatine and Louisa counties, Iowa. There, populations of *Kinosternon flavescens* and *Terrapene ornata* have been marked and released approximately every three years since 1973. This allows us to identify individuals and provide accurate assessment of age. This assessment is enhanced because we can accurately estimate age by counts of plastral annulae, for these species, usually to age 15. For very old box turtles, for example those whose plastrons were worn smooth in 1973 and are still alive with very deep plastral notches at the plastral hinges, estimates are crude suggesting ages beyond 75 and sometimes 100 years. Snapping turtle age was estimated by a combination of size and carapacal annulae counts. These too must be regarded as crude but are adequate for the comparisons being made in this study. Specimens were collected with the aid of 4,000 feet of drift fence with one trap approximately every 30 feet. The fence was placed so that it interrupted the path of the turtles as they moved to and from water. Cultures were started from skin samples taken by biopsy, usually of skin of the leg or tail. The turtle was released after the sample was taken. Rarely a turtle was sacrificed so that telomere length or telomerase analysis and cultures could be made of all the major organs.

Cultured Fibroblast Replications as an Indicator of Lifespan

Biopsied tissue was minced by the crossed scalpel method and sometimes cells were separated by trypsinization. Fibroblasts and other cells grown were cultured by the method developed in our laboratory for melanomacrophages (Rund et al, 1998). Cultures were passaged when they were near confluence. Cell counts and studies of morphology were conducted prior to each passage. A photographic record was kept of morphological changes, especially those associated with cellular senescence.

We checked for transformation by watching for a “crisis” decline in the cultures described by Finch (1990) that could precede transformation of a few cells and regrowth of the culture. Cell morphology was continuously studied for changes related to transformation.   We concluded that the culture would have terminated when the transformation occurred. Cultures were concentrated and snap frozen in liquid nitrogen at various stages in culture life for telomere length and telomerase analysis.

Studies of Telomere Length and Telomerase

Telomere repeat fragment analysis (TRF) for telomere length was performed by standard methods. Tissues were extracted with a Promega Genomic DNA extraction kit. Genomic DNA was digested by HaeIII (4-cutter) digestion. Fragments were separated by CHEF gel electrophoresis, transferred to MSI nylon membrane, and hybridized (Pharmagen Kit). Telomerase activity was measured in tissues and untransformed fibroblasts by the TRAP method of Lin et al (1998). Discovery of production of telomerase throughout the life of the untransformed fibroblast colony would provide evidence that long lived reptiles extend lifespan by production of telomerase. This would provide a natural vertebrate model for extension of vertebrate life through maintenance of activity of the telomerase gene.

RESULTS

Cultures completed for tissues of an approximately 16 year old mud turtle (*Kinosternon flavescens*) senesced in 1999 at 35 to 45 cell population doublings (cpds). Two cultures underway for a 21 year old mud turtle have senesced at 18-21 cpds. Most cultures from painted turtle JLC 6552 estimated to be 35 years old or older, are continuing to grow vigorously at 20 to 24 cpds. However those from a female judged to be the same age have senesced after 24 cpds. These brief observations are consistent with an intermediate lifespan of painted turtles of 50-70 years. All hatchling painted turtle cultures are still growing rapidly. Cultures from a 19 year old long lived turtle, *Terrapene ornata* are continuing to divide after 29 cpds. Those from a very old specimen (crudely estimated to be near 100 years old) showed very little cell division after the culture was started. The behavior of fibroblast cultures from a large, approximately 40 year-old snapping turtle are still dividing vigorously after 190 cpds. We see no evidence of transformation in these cultures and the behavior and appearance of the cells is entirely normal so far as we can determine at this time. Generation time for the snapping turtle culture has continued at 32-35 hours since inception. These cultures remain highly contact inhibited. Analysis for viral contamination is scheduled. We have not yet conducted studies of telomerase for any of the many snap frozen cultures set aside in the course of this work. A summary of current cell cultures is provided in Table 1.

We were able to culture many cells from the teiid lizard *Cnemidophorus sexlineatus.* All have now died except for hepatic fibroblasts that are still dividing after 20 cpds. This is surprising for a lizard that should be through 3/4 of its lifespan and may be a reflection of the telomerase we found in liver and some other organs of this species. Cultures from a one-year-old juvenile blur racer (*Coluber constrictor*) are continuing to divide after 42 cpds.

Telomerase analysis has been conducted on the major organs of one subadult specimen of *Chelydra serpentina*, one small adult male softshell turtle (*Apalone mutica*), two adult and two hatchling *Chrysemys picta,* Two hatchling and two adult *Terrapene ornata,* four *Cnemidophorus sexlineatus,* and one juvenile *Coluber constrictor*. The analysis was negative in all adult turtles and the snake for all organs except the gonad for which it was always positive. The analysis was positive for the liver and possibly spleen of only one of the two hatchling *Terrapene ornata*. The organs analyzed were usually skin, liver, spleen, kidney, gonad, skeletal muscle, heart, lung, and stomach.

Telomerase analysis for the juvenile blue racer, *Coluber constrictor* was negative but several organs of the lizard *Cnemidophorus sexlineatus* were strongly to weekly positive.

Repetition of the lizard analyses gave mixed results with usually the gonad and at least one other organ positive. These observations are suggestive that most organs of these lizards produce telomerase to a much greater extent than do other reptiles but further testing is required before conclusions can drawn. A one-year study of size classes in the Big Sand Mound population of *Cnemidophorus sexlineatus* suggests a maximum of four years survival in nature.

Analysis for telomere length suggested reductions of approximately 1/3 to 1/2 between hatchling *Kinosternon flavescens* and a 21 year-old adult. No difference was seen in comparison of hatchling and an approximately 20 year-old ornate box turtle. Adult-hatchling comparisons are not yet completed for other species and all of our experiments need several repetitions before we can consider the observations reliable.

DISCUSSION AND CONCLUSIONS

This preliminary study suggests that the yellow mud turtle, *Kinosternon flavescens* may serve as a typical model of reptilian growth and senescence. We have significant unpublished data indicating that this species undergoes senescent processes that are often similar to those experienced by mammals. Our field studies indicate no evidence that these turtles live more than 35 years. Our very preliminary data here suggest that fibroblasts taken from various organs undergo senescence by 45 population doublings and those removed from older turtles undergo significantly fewer doublings than those removed from young individuals. Telomeres in this species appear to shorten considerably between hatching and middle age (20-25 years). This study also indicates that post-embryonic individuals of this species, like most mammals studied, fail to produce significant telomerase in most organs. The gonad, analyzed with the other tissues as a positive control, always produces telomerase.

We have inadequate data to discuss most other turtles but some observations are worthy of comment. Our cultures of fibroblasts from snapping turtles appear to be immortal and untransformed, having exceeded 190 cpds with no indication of senescence. Our analysis of snapping turtle tissues indicate no telomerase except in the gonad where it is always present. Obviously, the telomere must be maintained by some mechanism. Possibly analysis of our snap frozen cultures await analysis. It is possible that occasional bursts of telomerase activity appear in the first year post hatching for ornate box turtles. Much more study is needed here and hatchling box turtles are hard to find in nature, at least in Iowa. If true, this mechanism could promote the long lifespan of this species.

Most of the large organs of the six-lined racerunner showed presence of telomerase in one analysis or another but the enzyme seemed not to be consistently present. The fact that almost every analysis found it in some organ other than the gonad suggests that this lizard differs from the other reptiles we examined. Cell replication studies of Simpson and Rausch (1989) found seemingly immortal tissues in the lizard *Anolis carolinensis* such as we may have observed in the snapping turtle. Our lizard studies are at this point inconclusive.

In general this study is only suggestive and we are in the process of repeating experiments and initiating new studies. This work certainly suggests that reptiles may reveal mechanisms that have implications for longevity and maintenance of tissue repair.

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Table 1. Tissues currently in culture for Hayflick studies.

Coll.#	Species	Tissue Source	Growth	Pasg	CPD	Culture ID	Date Started	Age	Comment
JLC6584	Kinosternon flavescens	Skin fibroblasts	Mod. Rapid	3	18	JLC6584-1	12-May-00	21 yrs	died senescence
JLC6584	Kinosternon flavescens	Skin fibroblasts	Slow	2	12	JLC6584-2	12-May-00	21 yrs	died senescence
JLC6632	K flavescens fml							35yrs	
	K. flavescens (h)								
JLC6552	Chrysemys picta ret. Male	testis	rapid	3	24	CpA	31-Aug-00	35+ yrs	
		spleen	rapid	2	20	CpA	31-Aug-00	35+ yrs	
		lung	rapid	3	23	CpA	31-Aug-00	35+ yrs	
		heart	no growth	4	20	CpA	31-Aug-00	35+ yrs	
		Skel muscle	rapid	2		CpA	31-Aug-00	35+ yrs	
JLC6636	Chrysemys picta (h)	Gonad	Rapid	3	24	CpJ	31-Aug-00	10 months	
		Skin fibroblasts	Rapid	2	11	CpJ	31-Aug-00	10 months	
	Chrysemys picta (h)	Skin fibroblasts	Rapid	9	27.5	CP-119	28-Apr-00	10 months	
	Chrysemys picta (h)	Skin fibroblasts	Slow	1	11	CP-17	28-Apr-00	10 months	
	Chrysemys picta (h)	Skin fibroblasts	Rapid	6	21	CP-125	28-Apr-00	10 months	
none	Chrysemys picta (h)	Spleen	Rapid	2	16	CP	7-Oct-00	1 month	
	Chrysemys picta (h)	Brain	Rapid	5	29		7-Oct-00	1 month	
JLC6543	Chrysemys picta feml	Liver	Senescent	6	24	C.picta	23-Feb-00	35+ yrs	Jaw cyst
JLC6630	Terrapene ornata (h)	Skin fibroblasts	Senescent	12	20	1,1-0L10R	28-Apr-00	1 yr, h lst yr	
JLC6631	T. ornata	Skin fibroblasts	apoptotic	2	12	6631		100 yrs	Smooth,v. deep st.
1,1-L10R	T. ornata	Skin fibroblasts	Senescent	9	29	1,1L10R		19 yrs	
JLC6542	Chelydra serpentina larg				190	JLC 6542	20-Jul-99	40 yrs	
JLC6638	Chelydra serpentina								
JLC6637	Cnemidophorus sexlineatus	Spinal cord	Senescent	1		C. sex ls.c.	31-Aug-00	3 yrs.	
	Cnemidophorus sexlineatus	Brain	Apoptotic			C. sex br	31 Aug 00	3 yrs	
	Cnemidophorus sexlineatus	Liver	Rapid	4	20	C. sex. liver	31 Aug. 00	3 yrs	
		Liver	Rapid				31 Aug. 00		small cells
		muscle	Apoptotic				31 Aug 00		
JLC6612	Coluber constrictor, Juv.	Skin fibroblasts	Rapid	13	42	6612 snake	30-May-00	10 months	

Figure 1. Left, young fibroblasts in culture; Right, senescent fibroblasts. Note the much larger cell size and lack of mitotic activity in the senescent fibroblasts.

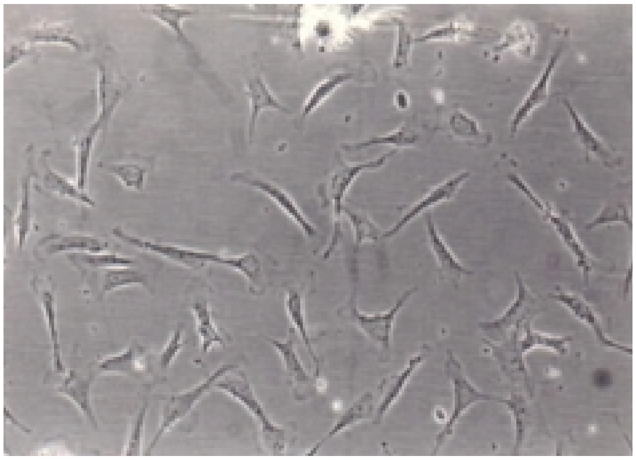

Figure 2. Telomerase analysis for liver and gonad of two specimens of *Cnemidophorus sexlineatus*. C= control. 1 and 3 = liver; 2 and 4 = gonad. Some of our earlier analyses showed greater or lesser amounts of telomerase in various organs of this species. The *Coluber constrictor* (snake) lanes 5 and 6, were damaged in this analysis.

C 1 2 3 4 5 6

— 50 bps

— 36 bps

Figure 3. Size classes of a population of *Cnemidophorus sexlineatus* measured in 1999 at Big Sand Mound in eastern Iowa. The September sample consisted only of hatchlings. The next highest band shows growth of sub adults. The top band is believed to consist of mostly three-year-old adults and some four year olds. This suggests a lifespan in nature of only four years. Surface activity of most of the large lizards ceases in August.

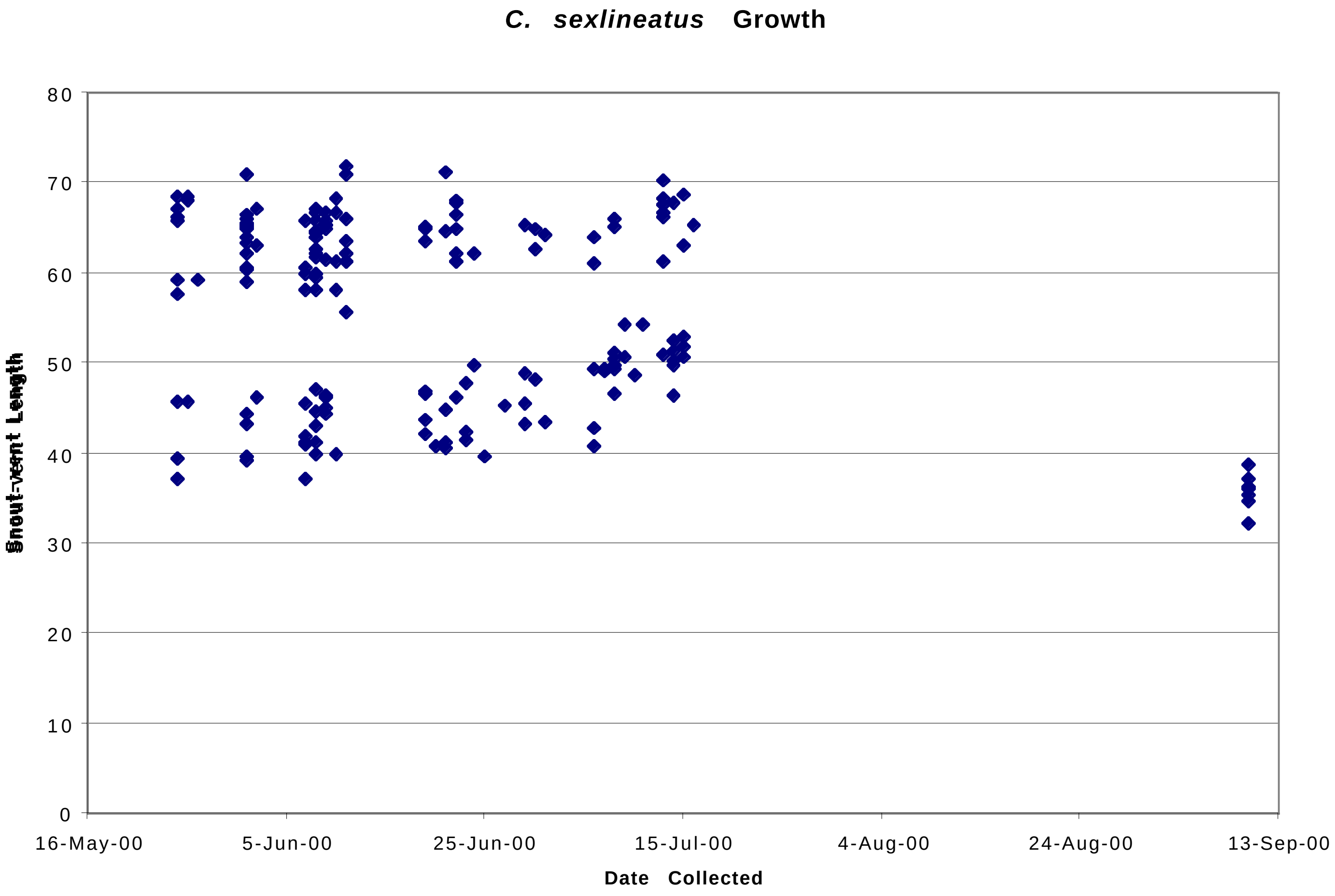

Figure 4. Telomere lengths of *Kinosternon flavescens* hatchling, lanes 2-4 and adult, lanes 6-8. The means of the telomere fragments for the adult are much smaller (1/3 – 1/2) those of the hatchling suggesting significant telomere loss with age for this species.