ANALYSIS OF RAT MICROGLIAL CELLULAR SENESCENCE
AS DETERMINED BY MEASUREMENTS OF
TELOMERE LENGTH AND TELOMERASE ACTIVITY

By

BARRY ERIC FLANARY

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2005
Copyright 2005

by

Barry Eric Flanary
I dedicate this research to my beautiful wife, Allison.
ACKNOWLEDGMENTS

I thank my mentor, Dr. Wolfgang Streit, who welcomed me as a researcher into his laboratory, permitted me to pursue the area of research most compelling to me, and who taught me an enormous wealth of knowledge. I also thank Dr. Gerry Shaw, Dr. Satya Narayan, and Dr. Jeff Harrison for serving on my dissertation committee and for their scientific advice. Appreciation is also extended towards my fellow lab colleagues, including: Chris, Amanda, Josh, Parker, Tanya, Nicole, Austin, Jackie, and Robert.

I thank Dr. Michael Fossel, who has helped open numerous doors of opportunity for me, for all of his help throughout the years, and for all that he has done for us, in particular for inviting me to be on the Editorial Board of his journal, *The Journal of Anti-Aging Medicine*, travelling to Illinois State University (when I was an M.S. graduate student there) to give a seminar on telomeres and cell senescence, and for inviting me to be a plenary speaker on his scientific panel at the Inaugural International Convention on Longevity in Sydney, Australia in March of 2004.

This thesis is written in honor of the late President Ronald Reagan (who succumbed to Alzheimer’s disease while I was working on this dissertation research), with the hope that a cure for the disease can be found sooner rather than later.

I especially thank my parents and brother for their support, and all that they have done to make it possible for me to pursue a path of science in life.

Special gratitude is expressed to my wife, Allison, for all of her patience, encouragement, and support while I was working on this dissertation research.
TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................................................ iv
LIST OF FIGURES .......................................................................................................... viii
ABSTRACT ..................................................................................................................... xii

CHAPTER

1 INTRODUCTION ........................................................................................................ 1
   Structure and Function of Telomeres ........................................................................ 1
   Characteristics of Cellular Senescence ................................................................. 2
   The Telomere Hypothesis of Cellular Aging .......................................................... 3
   Structure and Function of Telomerase .................................................................... 4
   Microglial Cells of the Central Nervous System ..................................................... 5
      Microglial Structure and Function in vitro and in vivo ....................................... 5
      Role of Microglia in Normal Brain Aging and Dementia .................................... 7
      Microglial Response Following Facial Motor Nucleus Axotomy ....................... 9
      Neuroprotective Functions of Microglia ............................................................. 11
   Specific Aims and Hypothesis ................................................................................ 12

2 PROGRESSIVE TELOMERE SHORTENING OCCURS IN CULTURED RAT MICROGLIA, BUT NOT ASTROCYTES ......................................................... 14
   Introduction ............................................................................................................. 14
   Materials and Methods .......................................................................................... 14
      Culturing of Microglia and Astrocytes ................................................................. 14
      Treatment of Microglial Cells ............................................................................. 16
      Determination of Telomere Length .................................................................... 16
      Determination of Individual Chromosomal Telomere Length ......................... 19
      Determination of Telomerase Activity .............................................................. 21
      Determination of Cell Proliferation and Viability .............................................. 23
   Results .................................................................................................................... 24
      GM-CSF Stimulates Microglial Proliferation ..................................................... 24
      Telomere Shortening Occurs in Cultured Rat Microglia .................................... 25
      Three-Fold Variation Exists in Individual Rat Microglia Telomeres ............... 30
      Cyclical Telomere Shortening Occurs in Rat Astrocytes ................................. 32
      Telomerase Activity in Cultured Rat Microglia and Astrocytes ....................... 38
   Discussion .............................................................................................................. 41
3 TELOMERES SHORTEN WITH AGE IN RAT CEREBELLUM AND CORTEX IN VIVO ..........................................................50

Introduction .................................................................................................................50
Materials and Methods ...............................................................................................50
  Collection of Rat Cerebellum and Cortex Tissues ..............................................50
  Determination of Telomere Length .................................................................50
  Determination of Telomerase Activity ..............................................................51
Results .........................................................................................................................51
  Telomeres Shorten With Age in Rat Brain in vivo ...........................................51
  Telomerase Activity in Rat Cerebellum and Cortex ..........................................51
Discussion ...................................................................................................................57

4 AXOTOMY INCREASES TELOMERE LENGTH, TELOMERASE ACTIVITY AND PROTEIN IN AXOTOMY-ACTIVATED MICROGLIA ...........................................61

Introduction .................................................................................................................61
Materials and Methods ...............................................................................................61
  Rat Facial Nerve Axotomy .................................................................................61
  FACS-Isolation of Rat Microglia from Micro-dissected Facial Nuclei ..............62
  Determination of Telomere Length .................................................................63
  Determination of Telomerase Activity ..............................................................63
  Telomerase Western Blot Analysis ......................................................................63
  Histochemistry ......................................................................................................64
  Statistical Analysis of Data ...................................................................................65
Results .........................................................................................................................65
  Increase in Microglia Surrounding Axotomized Facial Nuclei .......................65
  Increase in Telomere Length in Axotomized Facial Nuclei .........................66
  Increase in Telomerase Activity in Axotomized Facial Nuclei .......................69
  Increase in Telomerase Protein Quantity in Axotomized Facial Nuclei ..........72
  FACS-Isolation of Microglia from Facial Nuclei ..............................................74
  Increase in Telomerase Activity in FACS-Isolated Microglia From Axotomized Facial Nuclei .......................................................................................74
Discussion ...................................................................................................................78

5 ALPHA-TOCOPHEROL (VITAMIN E) INDUCES RAPID, NON-SUSTAINED PROLIFERATION IN CULTURED RAT MICROGLIA ........................................85

Introduction .................................................................................................................85
  Microglial Activation .........................................................................................85
  Function of Vitamin E ......................................................................................86
Materials and Methods ...............................................................................................87
  Culturing of Microglia .......................................................................................87
  Treatment of Microglial Cells ..........................................................................87
  Determination of Cell Proliferation ...............................................................88
  Determination of Interleukin-1β Production ....................................................88
  Determination of Telomere Length .................................................................88
Determination of Telomerase Activity ................................................................. 88
Statistical Analysis of Data .................................................................................. 88
Results .................................................................................................................. 89
  Microscopic Examination of Cultured Rat Microglia at Various Times and
  Treatments ............................................................................................................. 89
  Vitamin E Induces Cell Proliferation in Cultured Rat Microglia ......................... 89
  Telomere Length Analysis in Vitamin E-Treated Cultured Rat Microglia .............. 94
  Telomerase Activity Analysis in Vitamin E-Treated Cultured Rat Microglia ......... 95
  Interleukin-1 Beta Production in Cultured Rat Microglia .................................. 98
Discussion ............................................................................................................. 100

6 LIFE-SPAN EXTENSION IN NORMAL RAT MICROGLIA VIA
TELOMERASE REVERSE TRANSCRIPTASE RETROVIRAL
TRANSUDCTION .................................................................................................. 107

  Introduction ........................................................................................................ 107
  Materials and Methods ....................................................................................... 107
    Culturing of Microglia ...................................................................................... 107
    Production of Replication-Defective Telomerase-Encoding Retroviruses .......... 107
    Transduction of Rat Microglia With Telomerase-Encoding Retroviruses ........... 109
    Determination of Telomerase Activity ............................................................. 110
    Telomerase Western Blot Analysis .................................................................. 110
    Statistical Analysis of Data ........................................................................... 110
  Results ................................................................................................................ 110
    Telomerase-Encoding Retroviral Vector .......................................................... 110
    Telomerase Transduction Extends Life-Span of Microglia ............................... 111
    Telomerase Activity in Transduced Microglia ................................................ 113
    Telomerase Protein Quantity in Telomerase-Transduced Microglia ................ 117
Discussion ............................................................................................................ 119

7 CONCLUSIONS AND IMPLICATIONS .................................................................. 125

  Conclusions ....................................................................................................... 125
  Implications ....................................................................................................... 128

LIST OF REFERENCES ............................................................................................. 130

BIOGRAPHICAL SKETCH ....................................................................................... 146
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1. Cell proliferation in GM-CSF-treated cultured rat microglia as determined by MTT analysis</td>
<td>25</td>
</tr>
<tr>
<td>2-2. Southern blot analysis for measurement of telomere length in cultured microglia</td>
<td>27</td>
</tr>
<tr>
<td>2-3. Telomere length distribution in control and GM-CSF-stimulated microglia on days 1, 16, and 32</td>
<td>28</td>
</tr>
<tr>
<td>2-4. Southern blot analysis for measurement of telomere length in microglia cultured at varying densities</td>
<td>29</td>
</tr>
<tr>
<td>2-5. Telomere length distribution in microglia grown to near-confluence in various culture areas (9.5 cm(^2), 21 cm(^2), 175 cm(^2))</td>
<td>30</td>
</tr>
<tr>
<td>2-6. Telomere FISH analysis of metaphase spreads of cultured rat microglia using a FITC-conjugated peptide nucleic acid telomere-specific probe</td>
<td>31</td>
</tr>
<tr>
<td>2-7. Telomere fluorescence intensity (TFI) of all 168 individual telomeres in the 42 chromosomes of 2-day old cultures of rat microglia</td>
<td>32</td>
</tr>
<tr>
<td>2-8. Southern blot analysis for measurement of telomere length in non-passaged astrocytes from day 1 to 10</td>
<td>33</td>
</tr>
<tr>
<td>2-9. Telomere length distribution in non-passaged astrocytes from day 1 to 10</td>
<td>34</td>
</tr>
<tr>
<td>2-10. Southern blot analysis for measurement of telomere length in non-passaged rat astrocytes from day 2 to 32</td>
<td>35</td>
</tr>
<tr>
<td>2-11. Telomere length distribution in non-passaged rat astrocytes from day 2 to 32</td>
<td>36</td>
</tr>
<tr>
<td>2-12. Southern blot analysis for measurement of telomere length in astrocytes from passage 1 to 5</td>
<td>37</td>
</tr>
<tr>
<td>2-13. Telomere length distribution in astrocytes from passages 1 to 5</td>
<td>38</td>
</tr>
<tr>
<td>2-14. Telomerase activity in control (Con) and GM-CSF (CSF)-stimulated rat microglia on the indicated days</td>
<td>39</td>
</tr>
<tr>
<td>2-15. Telomerase activity in non-passaged rat astrocytes on the indicated days</td>
<td>40</td>
</tr>
</tbody>
</table>
2-16. Quantitation of telomerase activity (arbitrary units) in rat microglia and non-passaged astrocytes on the indicated days.................................................................41

3-1. Southern blot analysis for measurement of telomere restriction fragment (TRF) length in rat brain tissue .................................................................................................................................52

3-2. TRF length distribution in rat cerebellum and cortex samples on days 21 and 152..53

3-3. Average TRF length in rat cerebellum and cortex tissues on days 21 and 152........53

3-4. TRAP analysis for telomerase activity in rat brain tissue (days 21 to 182)........54

3-5. Quantitation of telomerase activity (arbitrary units) in rat cerebellum and cortex tissues (days 21 to 182)..........................................................................................................................55

3-6. TRAP analysis for telomerase activity in rat brain tissue (days 21 to 35)...........55

3-7. Quantitation of telomerase activity (arbitrary units) in rat cerebellum and cortex tissues (days 21 to 35)..........................................................................................................................56

3-8. Overall, the cerebellum exhibits higher telomerase activity than the cortex from day 21 to 35..............................................................................................................................56

4-1. Micrographs of axotomized (A) and control (B) facial nucleus on day 3 post-axotomy stained with GSI-B4 lectin to identify microglia .................................................................67

4-2. Southern blot analysis for measurement of telomere length in facial nuclei........68

4-3. Densitometric quantitation of telomere length in facial nuclei .........................69

4-4. Representative TRAP analysis image used for measurement of telomerase activity in facial nuclei. ..........................................................................................................................70

4-5. Densitometric quantitation of telomerase activity in facial nuclei ......................71

4-6. Densitometric quantitation of telomerase activity in unoperated facial nuclei ....72

4-7. Western blot image used for measurement of telomerase protein quantity in facial nuclei .................................................................................................................................73

4-8. Densitometric quantitation of telomerase protein in facial nuclei.......................73

4-9. FACS-isolation of microglia from axotomized and control facial nuclei ............76

4-10. TRAP analysis image used for measurement of telomerase activity in FACS-isolated facial nuclei.................................................................77

4-11. Densitometric quantitation of telomerase activity in FACS-isolated facial nuclei.................................................................................................................................77
5-1. Representative micrographs of cultured rat microglia under various treatment conditions .................................................................................................................90

5-2. Cell proliferation (as determined by MTT assay) of cultured rat microglia on the indicated days under various treatment conditions .................................................................91

5-3. Proliferation rate (as determined by BrdU incorporation over 2 hours) of cultured rat microglia on the indicated days under various treatment conditions .........................93

5-4. Proliferation of cultured rat microglia at 48 hours under various treatment conditions .................................................................................................................................94

5-5. Densitometric quantitation of telomere length in cultured rat microglia on the indicated days under various treatment conditions ........................................................................95

5-6. Representative TRAP analysis image used for measurement of telomerase activity in cultured rat microglia. ..................................................................................................97

5-7. Quantitation of telomerase activity in cultured microglia on the indicated days under various treatment conditions .................................................................................................98

5-8. Quantitation of telomerase activity in cultured microglia at 48 hours under various treatment conditions .................................................................................................................99

5-9: Quantitation of interleukin-1 β production by cultured rat microglia on the indicated days under various treatment conditions .................................................................99

6-1. The Clontech retroviral vector, pLPC-hTRT, used to transduce cultured rat microglia with the human telomerase reverse transcriptase (i.e., hTRT) gene ......112

6-2. Brightfield and green fluorescence micrographs of rat microglia and rat glioblastoma cells (RG-2) following retroviral transduction on day 4 ..............113

6-3. Representative micrographs of cultured rat microglia following retroviral transduction on days 1 and 20 ................................................................................................................114

6-4. Representative photographs of hTRT-transduced cultured rat microglia on days 57 and 75 .............................................................................................................................115

6-5. TRAP analysis image used for measurement of telomerase activity of cultured rat microglia on the indicated days under various transduction conditions ..........116

6-6. Quantitation of telomerase activity in cultured rat microglia on the indicated days under various transduction conditions .............................................................................................117

6-7. Western blot image used for measurement of telomerase protein in cultured rat microglia on the indicated days under various transduction conditions ..........118
6-8. Quantitation of telomerase protein quantity in cultured rat microglia on the indicated days under various transduction conditions.................................118
Normal somatic cells have a finite replicative capacity, and with each cell division, telomeres (the physical ends of chromosomes) progressively shorten until they reach a critical length, at which point the cells enter cellular senescence. Some cells maintain their telomeres by the action of the telomerase enzyme. Microglia, a non-neuronal cell type residing within the central nervous system (CNS), play vital roles in maintaining neuronal function, health, and survival in both the normal and pathological CNS. Microglia are the only adult cell type in the CNS that exhibit significant mitotic potential, suggesting that these cells have limited life-spans, may rely on proliferation to replace senescent cells, and are thus susceptible to telomere shortening and subsequent cellular senescence.

In our studies, we have found that telomere shortening occurred in cultured rat microglia concomitant with their progression to senescence by 32 days in vitro. Telomere shortening also occurred in vivo in both rat cerebellum and cortex from day 21...
to approximately 5 months of age (i.e., the oldest age analyzed). Axotomy-activated microglia from the facial nucleus (FN) maintained telomere length (TL) via increased levels of telomerase activity (TA) during periods of high proliferation in vivo. Microglia isolated directly from the axotomized FN via fluorescence-activated cell sorting exhibited increased TA relative to un-operated controls, suggesting that microglia are the primary cell type responsible for the increased TA observed in whole tissue FN samples. Vitamin E induced a significantly high proliferation rate in cultured rat microglia. This high rate of proliferation resulted in a concomitant decrease in TL, TA, and microglial activation. Microglia retrovirally-transduced with telomerase exhibited an increased maximal life-span (ranging from 230 to 375%), and delayed entry into senescence, relative to controls and empty-vector transduced microglia. Telomerase transduction did not immortalize microglia, although these cells exhibited a normal phenotype, and had telomerase activity/protein present well past the time when all control cells had died.

Our findings provide an impetus to further investigate rat microglial telomere dynamics in vivo, especially with age, following axotomy, or vitamin E supplementation, as well as in human microglia with age and incidence of Alzheimer’s disease.
CHAPTER 1
INTRODUCTION

Structure and Function of Telomeres

Telomeres are specialized structures at the physical ends of eukaryotic chromosomes consisting of essential proteins (e.g., TRF1, TRF2, TIN2, tankyrase) and highly conserved repeated DNA sequences (Kipling, 1995; Shay, 1999). Telomeres control genes near chromosome ends (Wright and Shay, 1992) and may direct chromosome attachment to the nuclear membrane (Gottschling and Cech, 1984). The very ends of telomeres, which contain about 10 to 20 nucleotides of single-stranded DNA, form telomere loops (T-loops) by means of a single-stranded DNA invasion event, and are thought to protect chromosome ends from degradation and end-to-end fusions (Shay, 1999). Vertebrate telomeres comprise the same sequence of hexanucleotide repeats (TTAGGG), (Moyzis et al., 1988). The length of telomeres is species-specific and ranges from 5 to 20 kilobases (kb) in humans (Harley et al., 1990) and from 20 to 150 kb in mice (Kipling and Cooke, 1990). Telomere loss occurs with each round of DNA replication (Harley et al., 1990) due to the inability of DNA polymerases to completely replicate linear DNA molecules (Olovnikov, 1971, 1973, 1996; Watson, 1972), and may also occur as a result of oxidative stress (von Zglinicki, 2002). Telomere length can be used as a predictor of the future replicative capacity of cells (Allsopp et al., 1992), and depends on both the age of the cell and the number of times the cell has already divided (Harley et al., 1990).
Characteristics of Cellular Senescence

Normal somatic cells undergo only a finite number of cell divisions in vitro before entering a non-dividing state called cellular senescence (Hayflick, 1961). Senescence can also occur in replication-independent manners, such as activation of p53 pathways (Shay et al., 1991) or when sufficient cellular damage accumulates. Senescence, which ultimately culminates in cell death, is characterized by an irreversible arrest of cell proliferation (Hayflick, 1965), substantial alterations in patterns of gene expression (i.e., SAGE: Senescence-Associated Gene Expression) (Bernd et al., 1982; Shelton et al., 1999; Funk et al., 2000), an increasing resistance to apoptosis (Spaulding et al., 1999), cell-type specific changes in cell function and gene expression (Funk et al., 2000), and concomitant telomere shortening (Harley et al., 1990). The reduction in proliferative capacity of cells from old donors (Bowman and Daniel, 1975) and patients with premature aging syndromes (e.g., Werner syndrome, and Hutchinson-Gilford progeria syndrome) (Prokof'eva et al., 1982), as well as the accumulation of senescent cells (Dimri et al., 1995) both in vitro and in vivo with altered patterns of gene expression (Shelton et al., 1999; Funk et al., 2000), implicates cellular senescence in aging and age-related pathologies (Fossel, 2000). After cells have exhausted their replicative capacity, they reach their Hayflick Limit and become incapable of further division (Hayflick, 1965). In some cells, a progression of intracellular events can lead to crisis (Wright et al., 1989), which is characterized by the appearance of one or more critically-short telomeres (Allsopp and Harley, 1995), which activate DNA-damaging signals (Harley, 1991), and cause end-to-end chromosomal fusions to occur (Cui et al., 2002). Thus, at least in vertebrates, it seems that the shortest telomere length, not the average, is responsible for maintaining chromosome stability, cell viability, and determining when a cell will enter
senescence (Hemann et al., 2001). At crisis, nearly all cells enter senescence and ultimately die by apoptosis (Payne et al., 1994), although some are able to up-regulate expression of the telomerase enzyme and become tumorigenic (de Lange, 1994). Following the senescence of a cell, replication of neighboring mitotic cells can occur, and their division to fill in the gaps left by senesced cells may cause their own telomeres to shorten in the process. Thus, senescence can lead to a propagating cycle of accelerated aging among remaining cells (Fossel, 2000).

**The Telomere Hypothesis of Cellular Aging**

The telomere hypothesis of cellular aging proposes that telomere shortening in mitotic somatic cells contributes to and causes their senescence, hastens the senescence of neighboring mitotic and post-mitotic cells (Harley et al., 1992), and underlies organismal aging (Fossel, 2000). This hypothesis (Harley et al., 1992) suggests that if telomeres in somatic cells can be maintained at/above, or increased to, pre-senescent levels (e.g., via telomerase) in order to prevent/reverse senescence, then replicative life-span should increase as well (Wright et al., 1996a; Fossel, 1998). If the life-span of individual cells can be increased, then as a result, the life-span of the entire organism may also be increased (Harley et al., 1992). Thus, if cell senescence can be slowed/prevented, then age-related diseases may also be slowed/prevented (Fossel, 1998, 2000). The presence of senescent cells may interfere with the normal functioning of, and may contribute to, organ and tissue aging (Dimri et al., 1995). Telomere shortening can be used as both an *in vitro* (Harley et al., 1990; Allsopp et al., 1992; Harley et al., 1992; Flanary and Streit, 2004) and *in vivo* (Lindsey et al., 1991; Kajstura et al., 2000; Wright and Shay, 2002; Flanary and Streit, 2003) marker of cell replication and cell aging. Telomere shortening can cause changes in expression of genes nearest the telomere (i.e.,
TPE: Telomere Position Effect) (Wright and Shay, 1992; Wood and Sinclair, 2002).
Thus, as telomeres shorten with age, genes (especially those nearest the telomere) can get over-expressed. Telomere position effect can result in the age-related expression and/or over-expression of genes near a telomere that is dependent on both distance from the telomere and individual chromosomal telomere length. It provides a mechanism for the modification of gene expression that occurs throughout the replicative life span of cells (Baur et al., 2001). The existence of TPE suggests that progressive loss of telomeres may lead to SAGE (Dimri et al., 1995; Fossel, 1998), which may affect both cell and organ function. Interestingly, some examples of human genes located nearest the telomere encode for well-known age-related diseases: cataracts, neuroblastoma, prostate cancer, Alzheimer’s disease, melanoma, obesity, colorectal cancer, ovarian cancer, diabetes, renal cell carcinoma, deafness, retinal degeneration, Huntington disease, leukemia, coronary artery disease, breast cancer, osteoporosis, glaucoma, deafness, emphysema.

**Structure and Function of Telomerase**

Elongation of telomeres can occur by the action of the ribonucleoprotein enzyme telomerase, which adds tandem hexanucleotide (TTAGGG)$_n$ repeats *de novo* to 3’ ends of mammalian telomeres using its own RNA as a template (Greider and Blackburn, 1985; Morin, 1989; Cech et al., 1997). Telomerase comprises two components: an RNA portion, which can be expressed in normal cells and is up-regulated during malignant transformation (Blasco et al., 1996), and a protein/catalytic portion, which is a reverse transcriptase expressed in rodent (Burger et al., 1997) and gametic/embryonic (Wright et al., 1996b) cell types, as well as during malignant transformation (de Lange, 1994). Telomerase can compensate for the continual shortening of telomeres that would otherwise occur in its absence. Elongation of telomeres can result in the extension of
cellular life-span (Wright et al., 1996a; Bodnar et al., 1998). Many diverse types of normal human and animal cell types have been transduced and subsequently immortalized with telomerase, such as bovine adrenocortical cells (Thomas et al., 2000), endothelial cells (Yang et al., 1999), epithelial cells (Bodnar et al., 1998), fibroblasts (Bodnar et al., 1998), keratinocytes (Guo et al., 1998), lymphocytes (Hooijberg et al., 2000), myoblasts (Seigneurin-Venin et al., 2000), osteoblasts (Yudoh et al., 2001), and pancreatic islet cells (Halvorsen et al., 1999). Reconstitution of telomerase (e.g., via retroviral transduction) in vitro into several diverse human and animal cell types can result in restoration of replicative potential, extension of telomere length and cellular life span, avoidance of cellular senescence (Bodnar et al., 1998; Vaziri and Benchimol, 1998), and reversion of gene expression to youthful levels (Funk et al., 2000) in the absence of tumorigenic changes (Belair et al., 1997; Jiang et al., 1999; Morales et al., 1999; Harley, 2002). During central nervous system (CNS) development, telomerase is highly expressed in neural progenitor cells, but sharply decreases as synapses form, and when cells undergo apoptosis or differentiate (Kruk et al., 1996; Mattson and Klapper, 2001).

**Microglial Cells of the Central Nervous System**

**Microglial Structure and Function in vitro and in vivo**

The central nervous system (CNS), which contains the brain and spinal cord, contain two main populations of cells: neurons and glia. Neurons are specialized cells important for relaying electrical signals to and from the brain and spinal cord. However, the majority of cells present within the CNS are not neurons, but glia. Glia (i.e., astrocytes, oligodendrocytes, microglia) provide structural, metabolic, and trophic support to neurons at all times. Microglia are distributed ubiquitously throughout the
central nervous system (CNS), and function as resident macrophages and antigen-presenting cells of the CNS (Thomas, 1992). They have vital roles in supporting and maintaining neuronal function, health, homeostasis, and survival in both the normal and pathological CNS microenvironment (Streit, 2002a, b) by phagocytosing amyloid β peptide (Frautschy et al., 1992), and secreting cytokines and neurotrophic factors (Streit et al., 1999; Nakajima et al., 2001; Streit, 2002a, b). Microglia have been aptly called “the brain’s immune system” because these cells share functional characteristics of cells in the peripheral immune system (e.g., lymphocytes and macrophages) (Streit and Kincaid-Colton, 1995). In addition to originating from bone marrow-derived hematopoietic progenitor cells (Eglitis and Mezey, 1997; Hess et al., 2004), microglia are capable of expressing MHC antigens, B- and T-cell lymphocyte markers, and other immune cell-specific antigens.

Unlike astrocytes and oligodendrocytes, microglia are capable of significant division, especially following neuronal injury (Kreutzberg, 1966; Graeber et al., 1988, Svensson et al., 1994). Following acute CNS injury, there is rapid activation of microglia and astrocytes. While acute microglial activation is marked by a conspicuous mitotic response, reactive astrocytes undergo primarily hypertrophy with markedly enhanced GFAP immunoreactivity, but show little mitosis (Graeber et al., 1988; Graeber and Kreutzberg, 1986; Kreutzberg, 1996). Thus, the mitotic ability of microglia in vivo is much greater than that of astrocytes. Interestingly, when these glial cell populations are maintained in vitro, the mitotic potential of astrocytes exceeds that of microglia, and astrocytes spontaneously form confluent monolayers that resemble those formed by cultured fibroblasts. Microglia, on the other hand, require stimulation with hematopoietic
growth factors to undergo significant cell division in vitro (Giulian and Ingeman, 1988; Suzumura et al., 1990). However, the mitotic potential of microglia both in vitro and in vivo suggests that these cells may rely on proliferation and self-renewal to replace senescent cells, and thus may have limited cellular life-spans.

Role of Microglia in Normal Brain Aging and Dementia

The role of microglial cells in the aging CNS and in the development of age-related neurodegenerative disease remains unknown. Alzheimer’s disease (AD) is an age-related, progressive neurological disorder characterized by significant memory loss, extracellular amyloid plaque deposition, intracellular neurofibrillary tangle formation within neurons, loss of neuronal synapses, the dysfunction and death of significant numbers of neurons, and memory loss (Mann and Yates, 1981). AD currently afflicts 1 in 10 individuals over age 65 and nearly half of those over age 85, with the incidence rate doubling approximately every 4.4 years after age 60 (Kawas et al., 2000). Microglial cells are known to be clustered around amyloid beta (Aβ)-containing senile plaques in the aged and AD brain (Itagaki et al., 1989), and this clustering likely occurs because the cells are gathering there in an attempt to remove insoluble deposits of Aβ (Frautschy et al., 1992). However, clearance of Aβ is often not achieved, and this raises the possibility that the Aβ clearing ability of microglia may be weakened or lost with aging. This may explain why substantial deposits of amyloid plaques can be found in elderly non-demented individuals (Dickson et al., 1992). In addition, there may be overproduction of Aβ such that microglia are overwhelmed by a larger-than-normal amyloid burden, which may compromise the ability of microglia to clear amyloid, and impair their other vital neuroprotective functions (Streit, 2002b).
Studies conducted in post-mortem human brains have shown an increased incidence of microglial cytoplasmic structural abnormalities (i.e., cytoplasmic swelling, twisted and shortened processes, and cytoplasmic fragmentation) and dystrophy in the cerebral cortex of aged and AD-diseased brains (Streit et al., 2004b), which support the hypothesis that microglia may become dysfunctional with age and that microglial dystrophy may contribute to their senescence, which in turn, may impair their neuron-sustaining functions and ultimately lead to neuronal cell death. It is reasonable that the increased presence of dystrophic microglia in elderly individuals occurs because the cells’ ability to divide is declining as a result of aging (replicative senescence) thereby slowing the replacement of senescent (dystrophic) microglia with younger cells. Based on these observations, we hypothesize that a reduced ability of microglia to clear amyloid with age and incidence of AD may be the result of their cellular senescence.

Understanding AD is complex and multi-faceted. Synapse loss is a hallmark characteristic of declining memory function with aging and may be linked to an impairment of neuronal and/or glial cell function. Neuronal integrity and function, in turn, are highly dependent on the presence of fully functional glial cells. In the normal CNS, microglia are engaged in the continuous monitoring of neuronal well-being (Streit, 2002b). To ensure proper neuronal functioning, complex molecular and cellular interactions occur between neurons and microglia. Since microglia are capable of producing both neuroprotective and neurotoxic molecules depending on the type of signals received from neurons (Streit et al., 1999), any impairment in microglial function due to cellular senescence (or otherwise) could have profound consequences for neuronal activity and cognitive function in the normal aging brain. Over time, microglia may enter
senescence and be less able, or unable, to maintain neuronal health. As a result, when sufficient quantities of microglia have begun to senesce, the neurons they once supported may begin to degenerate, enter senescence, and ultimately die as well due to diminished glial support and maintenance. Neuronal cell death leads to loss of communication and synapses between neighboring neurons, and ultimately is the cause of memory loss evident with age and in AD. Thus, neurodegenerative changes may occur because microglia are becoming senescent and dysfunctional, and as a result, may inadvertently contribute to neurodegeneration due to impaired glial support.

Neuronal cell death is a hallmark characteristic of AD and may be linked to an impairment of microglial cell function. Thus, understanding how microglia are involved in age-related deterioration of neuronal function is important for enabling the prevention of AD. A demonstration of microglial senescence with age would suggest that slow and progressive neurodegeneration and associated neuronal cell death, which are ultimately responsible for memory loss and dementia, may result from diminished or impaired microglial cell function. This could lead to the development of new drugs designed to enhance microglial cell function and/or to slow microglial telomere shortening and senescence as potential treatments of AD for humans.

**Microglial Response Following Facial Motor Nucleus Axotomy**

Even though the CNS is generally considered a post-mitotic tissue, it is important to note that microglia do retain a robust proliferative potential, especially under conditions of CNS injury (e.g., axotomy), as shown by DNA labelling studies using 3H-thymidine or bromodeoxyuridine (BrdU) (Graeber et al., 1988; Kreutzberg, 1966, 1996; Streit and Kreutzberg, 1988; Svensson et al., 1994). During facial nerve axotomy, the facial nerve is cut outside the brain and the reactions of facial motor neurons and their
Glial environment can be studied in the brainstem (Graeber et al., 1988; Kreutzberg, 1996). In the adult rat, unilateral axotomy of the facial nerve produces a robust, well-characterized microglial response within the ipsilateral facial motor nucleus. Since the contralateral facial nucleus is surgically unaffected, it serves as an internal control. In adult rats, by approximately 4 weeks post-axotomy, the motor neurons of the facial nucleus regenerate their functional connectivity (Kreutzberg, 1996), as noted by regained whisker movement (Streit, 1996). In addition, axotomy of the facial nerve does not disturb the blood–brain barrier (Raivich et al., 1998). After transection of the facial nerve, microglia but not astrocytes proliferate (Graeber et al., 1988), become hypertrophic, and express several cell surface molecules, such as complement receptor 3, major histocompatibility complex (MHC) classes I and II (Streit et al., 1989), co-stimulatory molecules (e.g., B7-1) (De Simone et al., 1995), and several cell adhesion molecules (Moneta et al., 1993). Microglial cells are activated and increase in number in the facial nucleus following peripheral axotomy. Microglia become motile and migrate towards the injured motor neurons within the axotomized facial nucleus, and microglial phagocytosis of bacteria can be observed in situ following axotomy (Schiefer et al., 1999). Within a few days following axotomy, microglia also maintain close contact with neurons and move along their dendrites, suggesting a possible role for microglia in "synaptic stripping", the displacement of afferent synaptic terminals from the motoneuron surface following axotomy (Kreutzberg, 1996; Schiefer et al., 1999). Under conditions of facial-nerve axotomy, facial motor neurons survive and will eventually regenerate their injured axons (Kreutzberg, 1996). Age does not affect the glial response to axotomy in regards to expression of glial fibrillary acidic protein (GFAP), leukocyte common
antigen, type 3 complement receptor, and MHC classes I and II (Hurley and Coleman, 2003). The fact that microglia undergo proliferative bursts shortly after an acute injury suggests that mitosis affords a mechanism to provide greater numbers of microglial cells, and thus increased trophic support, during CNS injury and distress. However, their ability to divide also suggests that the life span of microglia may be limited, and makes them susceptible to replicative senescence (Flanary and Streit, 2004).

**Neuroprotective Functions of Microglia**

Microglia play both neuroprotective and immunocompetent roles which serve to maintain neuronal health (Streit et al., 1999; Streit, 2002b). Neurons are especially fragile cells, and their well-being and proper functioning are highly dependent on the presence of large numbers of microglia that sustain a plethora of neuron-supporting functions. Microglia are extremely sensitive to even minor disturbances in CNS homeostasis and rapidly become activated and proliferate vigorously following nearly all neuropathologic conditions, such as nerve injury, stroke, and trauma (Streit et al., 1999). Glial activation after injury is a beneficial and ostensibly necessary process, and serves not only to restore homeostasis within the CNS microenvironment but also to assist in the regeneration of injured neurons.

In addition to protecting the CNS from invading microorganisms, microglia are important also for providing neuroprotection to normal and damaged neurons. Therefore, it is important to sustain a healthy microglial population in order to help keep the CNS functioning properly. During times of increased stress (e.g., acute neuronal injury), microglia are especially important due to their unique ability to rapidly respond to neuronal injury via migration, proliferation and trophic factor production. The observation that there are as many microglia in the brain as there are neurons (Streit and
Kincaid-Colton, 1995), in conjunction with the fact that microglia represent the only type of mature brain cells capable of undergoing mitosis and self-renewal, emphasizes the importance of these cells for providing constant monitoring of neuronal well-being and targeted trophic support to neurons that may encounter acute stress situations.

Following axotomy of motor axons within the facial nerve, neuronal survival and axonal regeneration is accompanied by vigorous microglial activation and cell proliferation. Thus, microglial activation, which begins long before axons have regenerated and serves to assist in the regeneration of injured motor neurons, is an integral, and potentially crucial, component of the regeneration process. Since axotomized motor neurons do regenerate, the rapid onset of microglial activation likely occurs because injured neurons are recruiting nearby microglia to assist them in their struggle to survive and regenerate (Streit et al., 1999). These observations strongly support a neuroprotective and pro-regenerative role of microglia in the injured CNS.

**Specific Aims and Hypothesis**

The specific aims are as follows:

1. To determine if cultured rat microglia are subject to telomere shortening and senescence when cultured *in vitro*.
2. To determine if telomere shortening occurs in the rat brain with aging.
3. To determine if neuronal injury-induced microglial proliferation within the facial nucleus resulted in telomere shortening *in vivo*.
5. To determine whether exogenous delivery of the telomerase gene via retroviral transduction could prevent microglial senescence and extend life-span of cultured rat microglia.

Collectively, these experiments have focused on studying the hypothesis that with aging, microglia undergo telomere shortening both *in vitro* and *in vivo*, become
increasingly dysfunctional, and ultimately enter cellular senescence. The rationale for this hypothesis is based on the fact that microglia undergo cell division \textit{in vivo}, and are thus susceptible to telomere shortening with age. If this situation does indeed occur \textit{in vivo} in multicellular telomerase-negative organisms (e.g., humans), it may lead to a decline in microglial cell function with age, which in turn, would inhibit their ability to promote neuronal well-being. Thus, age-related neuron loss may be due to loss of microglial support.
CHAPTER 2
PROGRESSIVE TELOMERE SHORTENING OCCURS IN CULTURED RAT MICROGLIA, BUT NOT ASTROCYTES

Introduction

To study the possibility that microglial cells in vitro are subject to replicative senescence, we decided to investigate telomere shortening and telomerase activity in microglia. We now present evidence to show that progressive telomere erosion occurs in cultured rat microglia, while astrocytes exhibit a cyclical pattern of telomere shortening and lengthening.

Materials and Methods

Culturing of Microglia and Astrocytes

Microglia were isolated from newborn Sprague-Dawley rat brains. The cerebral cortices of neonatal rats (≤ 3 days) were stripped of meninges and minced with a sterile scalpel blade in a 35 x 10 mm dish containing filter-sterilized 37°C solution D (0.137 M NaCl, 0.2 M NaH₂PO₄, 0.2 M KH₂PO₄, 5.4 mM KCl, 5 mM dextrose (glucose), 58.5 mM sucrose, 0.25 μg/mL Fungizone (Gibco, Carlsbad, CA), and 1 x 10⁶ U penicillin/streptomycin in sterile water). The tissue fragments/cell suspension were incubated in 37°C solution D containing 1.0% trypsin (Invitrogen, Carlsbad, CA) for 30 min. at 37°C on a bi-directional tilting platform. An equal volume of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin (complete medium) was added to quench the trypsin reaction. The mixed brain cell suspension was then passed through a 130 μm Nitex filter.
(Tetko, Inc., Briarcliff Manor, NY) and centrifuged (4,000 rpm (2,900 g), 10 min). The resulting pellet was resuspended in 10 mL of complete medium, passed through a 40 μm Nitex filter, and plated on poly-L-lysine (0.01 g/L) (Sigma-Aldrich, St. Louis, MO) coated, solution D-rinsed, 175 cm² flasks at a density of 1.5 brains per flask. The cultures were incubated in complete medium at 37°C under 5% CO₂. After 4 days, the medium was changed and incubation was continued for an additional 3 days. Microglia were harvested from the whole brain cultures by shaking the flasks on an orbital shaker (100 rpm) for 1 hour (which detached the loosely-adherent microglia), and then collecting the medium containing the free-floating microglia. The cells were then pelleted from the medium by centrifugation (4,000 rpm (2,900 g), 10 min), resuspended in fresh complete medium, and immediately plated (day 0) in cell culture dishes at the appropriate cell concentrations as follows: 9.5 cm² plates (1.0 x 10⁶ cells/well), 3.8 cm² plates (4.0 x 10⁵ cells/well), or 0.32 cm² plates (3.4 x 10⁴ cells/well). The optimal initial cell plating density was empirically determined in previous experiments. Cells were allowed to settle for 1 hour in a 5% CO₂, 37°C incubator, and then the culture medium was changed to remove any contaminating non-adherent cells. The microglia were then treated with the appropriate concentration of a particular treatment regimen.

Astrocytes normally form monolayers that cover the bottom of the culturing flask containing mixed brain cell cultures. To prepare enriched astrocyte cultures, all cells adhering to the astrocytic monolayer were detached by vigorously shaking the flasks at 200 rpm for 1 hour. The culture medium containing the floating cells was then removed, and the remaining adherent astrocytes were rinsed with PBS, trypsinized, counted, and plated. Astrocytes were plated on day 0 at an initial density of 1x10⁶ cells/well in 9.5
cm² plates and allowed to divide, or at a density of 2x10⁶ cells in 175 cm² flasks and passaged when confluent.

**Treatment of Microglial Cells**

Microglia were treated on day 0 with either 1.0 nM (0.015 μg/mL) or 10.2 nM (0.15 μg/mL) recombinant rat granulocyte-macrophage colony stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN), 100 nM lipopolysaccharide (LPS), or received no stimulation (control). In all experiments, media (and respective treatment) were changed as needed (usually every 3 to 4 days).

**Determination of Telomere Length**

To measure telomere length (i.e., telomere restriction fragment (TRF) length: the length of the telomere plus sub-telomeric DNA, the latter being dependent upon the particular cleavage sites of the two restriction enzymes used), Southern blot analysis using chemiluminescent detection and the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Indianapolis, IN) was employed as previously described (Flanary and Streit, 2003, 2004), with minor modifications. Genomic DNA was isolated from 1) cultured cells at various time points, 2) whole brain tissue samples, or 3) pooled micro-dissected facial nuclei (approximately 7 mg wet weight each), using the DNeasy DNA isolation kit (Qiagen, Valencia, CA). DNA concentration was determined by absorbance at 260 nm, while DNA purity was calculated by the ratio of 260/280 nm absorbance, using a spectrophotometer. For Southern blotting, either 2.5 μg (for cultured cells), or 5.0 μg (for tissues) of DNA was digested with 10 units each of *Hinfl* and *RsaI* overnight at 37°C. Following digestion, 5.0 μL gel loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol) was added to each sample. Each digested DNA
sample, and two samples containing a digoxigenin-labeled molecular weight (MW) marker (100 ng each lane) (i.e., DIG DNA MW marker II) (Roche, Indianapolis, IN), were then loaded onto a horizontal 15 x 25 cm 0.5% agarose gel, and electrophoresed at 70 volts in 1X TBE buffer at 4°C with buffer recirculation until the bromophenol blue band ran off the gel and the xylene cyanol band reached 80% the length of the gel (approximately 21 hours). The gel was then soaked successively in depurination solution (0.25 M HCl) for 5 min, then denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 2 x 10 min., and finally in neutralization solution (1.5 M NaCl, 0.5 M Tris) for 2 x 10 min. Unless otherwise noted, all incubations and washes were performed at room temperature with gentle agitation. The gel was rinsed three times in sterile double-distilled water after each treatment noted above, and was then equilibrated in 20X SSC (3.0 M NaCl, 0.3 M sodium citrate; pH 7.0) for 10 minutes before the DNA was vacuum blotted (Boekel, Feasterville, PA) onto a positively-charged nylon membrane at 45 mbar for 45 min. Following UV-crosslinking (120 mJ/cm²), a 2 min. wash in 2X SSC, and prehybridization at 41°C for 1 to 2 hours, hybridization of telomeric repeats was accomplished by using a digoxigenin-labeled telomere-specific oligonucleotide probe (TTAGGG)₃. Digoxigenin labeling of the probe (100 pmol) was accomplished by using the DIG oligonucleotide tailing kit (Roche, Indianapolis, IN). Following hybridization at 41°C overnight (approximately 17 hours) in a hybridization oven (Hybaid, Franklin, MA) with gentle rotation, the membrane was washed in 2X wash buffer (2X SSC, 0.1% SDS) for 2 x 5 min., followed by washes in 0.5X wash buffer (0.5X SSC, 0.1% SDS) for 2 x 15 min. at 55°C in a hybridization oven with moderate rotation. After washing the membrane in washing solution (0.1 M maleic acid, 0.15 M NaCl, 0.3 % (v/v) Tween-20;
pH 7.5) for 2 min., the membrane was then soaked in blocking solution for 1 to 2 hours, followed by incubation with a 1:10,000 dilution of an alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody for 30 to 45 min. Following washes in washing solution for 2 x 15 min., chemiluminescent detection was accomplished by washing the membrane in detection solution (0.1 M NaCl, 0.1 M Tris; pH 9.5) for 3 min., and then incubating the membrane (wrapped in plastic wrap) in the AP-metabolizing substrate CDP-Star (Roche, Indianapolis, IN) for 5 min. at 37°C. Following exposure of the membrane to X-omat AR Film (Eastman Kodak Company, Rochester, NY) in an autoradiography cassette for 1 to 60 min., the film was developed using a Konica SRX-101A automatic film processor (Konica Minolta, Mahwah, NJ). A digital image of the autoradiograph was generated by scanning it using a GS-710 calibrated imaging densitometer (BioRad, Hercules, CA). Telomere lengths (shortest, mean, longest) of each sample were calculated by comparison to known MW standards present on the gel (in each outside lane), and quantified using the computer program Telometric (version 1.2) (Grant et al., 2001).

Telomere lengths (shortest, mean, longest) of each sample were determined as follows. The length of the “shortest” telomeres represented the telomere signal (smear) on the autoradiograph corresponding to the smallest telomeres (lowest MW), which was calculated by measuring the bottom of the smear in each lane. Similarly, the length of the “longest” telomeres represented the signal corresponding to the longest telomeres (highest MW), which was calculated by measuring the top of the smear in each lane. The length of the “mean” telomeres represented the signal corresponding to the average length of all telomeres within the entire length of the smear. Telomere length
measurement by current Southern blot techniques normally, and unavoidably, incorporates sub-telomeric DNA regions into the calculated MW due to the use of DNA digestion via restriction enzymes. Sub-telomeric DNA regions are located directly adjacent to the telomere regions of DNA, and are still connected to the telomere region during gel electrophoresis due to the action of restriction enzymes, which do not cleave directly at the telomere/sub-telomeric region. During gel electrophoresis, the presence of these small sub-telomeric DNA regions will slow the migration of telomere regions, which would otherwise migrate slightly faster in the absence of such attached sub-telomeric regions. Thus, all protocols used for Southern blot for telomere length analysis utilizing restriction enzymes do not generate a “true” measurement of “actual” telomere length, since the pure telomere DNA region (if unconnected to adjacent sub-telomeric regions) would migrate faster on the gel, and thus would have a slightly smaller actual MW than what was measured.

**Determination of Individual Chromosomal Telomere Length**

Fluorescence *in situ* hybridization (FISH) is a molecular cytogenic technique that is used to obtain information from metaphase (Poon et al., 1999) or interphase (De Pauw et al., 1998) cells, depending on the specific sequence of the fluorochrome-conjugated probe applied. We used a telomere-specific FITC-conjugated probe, and the binding of the probe to its target (telomeres) can be identified by a distinct green fluorescence signal at the tips of metaphase chromosomes. For FISH analysis, metaphase chromosomes were obtained from cultured microglia using standard methods. Briefly, fresh cultures (day 0) of microglia were stimulated to divide with 10 nM rrGM-CSF for 2 days on top of glass coverslips. Colchicine (10 μg/mL) was added, and the cells were incubated for 1 hr. at
37°C. This step disrupts and prevents formation of mitotic spindles, prevents completion of mitosis, and enriches the population of metaphase cells. Following aspiration, pre-warmed (to 37°C) 0.075 M KCl was added, which makes nuclei swell osmotically and helps prevent chromosome overlap, and the cells were incubated for 20 min. at 37°C. The cells were then fixed with ice-cold methanol/acetic acid (3:1). A FITC-conjugated peptide nucleic acid (PNA) telomere-specific probe (Dako, Carpinteria, CA) was added, which was used due to its high sensitivity and specificity. PNA is a synthetic DNA/RNA analog capable of binding 99 to 100% of telomere repeats. Additionally, this probe does not recognize subtelomeric sequences and, therefore, will allow for an exact measurement of telomere length. Chromosomes were counterstained with 100 mg/mL propidium iodide and mounted with the antifade reagent Vectashield. The preparations were viewed with both a Zeiss Axioskop 2 fluorescence microscope connected to an RT color Spot digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) using a 60X and 100X oil lens, and a Bio-Rad 1040 ES confocal system connected to an Olympus IX70 inverted microscope using an Olympus planapo 60X 1.40 oil lens. High quality preparations were photographed using a digital camera at 1024 x 1024 resolution. Using special software, telomere length was measured at the ends of individual chromosomes from digital images of metaphase spreads (Poon et al., 1999) using the Zeiss fluorescence microscope. Using Image-Pro Plus software (Media Cybernetics, Carlsbad, CA), individual telomere fluorescence intensity was measured. This enabled a determination of whether intrachromosomal telomere length variation occurs in these cells, and if certain chromosomes are more susceptible to shortening over time.
Determination of Telomerase Activity

Telomerase activity was measured using the telomere repeat amplification protocol (TRAP) as previously described (Flanary and Streit, 2003, 2004), with minor modifications. Total protein was isolated from 1) PBS-washed cultured cells, 2) whole brain tissue samples, or 3) pooled micro-dissected facial nuclei (approximately 0.5 mg wet weight each), using 200 μL CHAPS lysis buffer (5.0 mM β-mercaptoethanol, 1.0 mM EGTA, 1.0 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM Tris, 0.5% CHAPS, 10% glycerol). The protein extract solution was collected in RNAse-free tubes, incubated on ice for 30 min., and centrifuged at 12,000 g (14,000 rpm) for 20 min. at 4°C to sediment residual cell debris, if present. Protein extracts were then aliquoted into RNAse-free tubes and stored at -80°C. Immediately prior to TRAP analysis, total protein concentration was measured using the BCA protein assay reagent (Pierce, Rockford, IL) as per the manufacturer’s recommended protocol. For TRAP analysis, each sample set included normal protein extracts, a telomerase-negative control (CHAPS lysis buffer, and/or RNAse-treated extracts: 10 mg/mL RNAse:sample (1:1) incubated for 20 min. at room temp.), and a telomerase-positive control (500 ng protein extract of a rat glioblastoma cell line RG-2). Each 50 μL reaction initially contained 5.0 μL 10X TRAP buffer (10 mM EGTA, 500 mM KCl, 15 mM MgCl₂, 100 mM Tris, 1.0 mg/mL BSA, 0.05% Tween-20), 200 μM dNTP (Roche, Indianapolis, IN), 100 ng telomerase substrate (TS) primer (5’-AAT-CCG-TCG-AGC-AGA-GTT-3’), 500 ng protein extract, and RNAse-free water up to 48 μL. This mixture was incubated for 20 min. at room temperature to allow telomerase, if present and active, to add hexanucleotide telomeric repeats (i.e., TTAGGG) onto the 3’ end of the TS primer, which is a substrate
oligonucleotide and served as an artificial telomere. Following telomeric extension, 100 ng CX primer (5’-CCC-TTA-CCC-TTA-CCC-TTA-CCC-TAA-3’) and 5 units Taq polymerase (Fisher Scientific, Pittsburgh, PA) were added. Telomere repeats were amplified by the polymerase chain reaction (PCR) using the TS (forward) and CX (reverse) primers, which generate a 40 base pair internal control band (i.e., TS-CX primer dimer) in each lane (including the negative control lane, since the presence of the internal control band is independent of the activity of telomerase), and a ladder of products (generated by telomerase) containing 6-base increments beginning at 46 base pairs in telomerase-positive lanes. Both an increased quantity and intensity of bands present within the ladder of products correspond to an increased level of telomerase activity. If telomerase activity was absent, no product ladder was formed and only the internal control band was evident. PCR was carried out as follows: Initial denaturation at 94°C for 2 min. to inactivate telomerase, then 33 total cycles of the following: 94°C for 30 sec., 55°C for 30 sec., 72°C for 45 sec. Following PCR, 5 μL filter-sterilized gel loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 50% glycerol, 50 mM EDTA) was added to each sample. The samples were then loaded onto a vertical 20 cm 12.5% non-denaturing polyacrylamide gel, and electrophoresed at 87 volts at room temperature in 0.5X TBE buffer until the bromophenol blue band ran off the gel and the xylene cyanol band reached 95% the length of the gel (approximately 21 hours). The telomerase products were visualized by staining the gel with a 0.01% solution of SYBR Green (Molecular Probes, Eugene, OR) for 40 min. in the dark with gentle agitation, and then photographing the gel under ultraviolet light using an electronic gel documentation system (Gel Doc 2000, BioRad, Hercules, CA). Quantitation of telomerase activity (i.e.,
the ladder of products formed in each lane) was performed using the densitometry computer program Quantity One (version 4.3.1) (BioRad, Hercules, CA). Average telomerase activity was determined by calculating the mean of individual quantitative measurements from two or more identical samples. Normalized telomerase activity was determined by comparing the average densitometric values of identical samples run on different gels in order to make an accurate comparison of telomerase activity between all samples run on multiple gels.

**Determination of Cell Proliferation and Viability**

To assess cell proliferation, two methods were employed: the colorimetric MTT assay, and 5-Bromo-2’-deoxyuridine (BrdU) incorporation. For the MTT assay (Boehringer Manheim, Indianapolis, IN), which measures both cell proliferation and cell viability, microglia were plated at an initial density of $3.4 \times 10^4$ cells/well in 0.32 cm$^2$ (96-well) plates. MTT labeling reagent (5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide in PBS) (Boehringer Manheim, Indianapolis, IN) was added (10 μL per well) to the cultured cells at various time points. Metabolically-active cells cleave the yellow tetrazolium MTT salt to form purple formazan crystals via NADH reductase. Following a 4 hour incubation at 37°C under 5% CO$_2$, cells were solubilized overnight in 10% SDS in 0.01 M HCl (100 μL per well). The solubilized formazan product was spectrophotometrically quantified at 550 nm (using a reference wavelength of 655 nm) using a Benchmark microplate reader (BioRad, Hercules, CA) and microplate manager software (version 4.0).

To determine proliferation using BrdU incorporation, microglia were plated at an initial density of $2.0 \times 10^5$ cells/well in 1.9 cm$^2$ plates (24-well plates). BrdU (Sigma-
Aldrich, St. Louis, MO; catalog # B5002) was added (10 μM final concentration) to the cultured cells at various time points. Proliferating cells incorporated the BrdU (in place of thymine) during S-phase. Cells were fixed at 4°C overnight in 80% EtOH. The following morning, wells were rinsed with PBS and stored at 4°C in PBS until assayed. For BrdU analysis, each plate was incubated for 10 minutes at 37°C in 2M HCl. Following washes in PBS for 3 x 5 min., blocking buffer (PBS containing 0.1% Triton X-100 and 2% normal goat serum) was added, and each plate was incubated for 30 min. at 37°C. A FITC-conjugated rat anti-BrdU antibody (Serotec, Raleigh, NC; catalog # MCA-2060-FT) diluted in blocking buffer was added to each well, and each plate was incubated for 2 hours at room temperature covered with foil (to prevent degradation of fluorescent signal). Wells were rinsed with PBS, and cell nuclei were stained with a 1.0 μg/mL solution of 4’-6-Diamidino-2-phenylindole (DAPI) for 5 min. covered with foil. Cells were photographed under fluorescence using a digital camera (Sony DSC-S75 Cyber-shot, 3.3 megapixels, Carl Zeiss Vario-Sonnar lens) connected to a Zeiss Axiovert 25 fluorescence inverted microscope.

Cell viability was performed on cultured microglia using the live/dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR).

Results

GM-CSF Stimulates Microglial Proliferation

Microglia exposed to GM-CSF increased proliferation compared to controls (Fig. 2-1). Cell viability analysis of cultured microglia indicated that with increasing time in culture, the total number of viable cells decreased in both control and GM-CSF-treated groups, indicating that the cells were entering replicative senescence (data not shown).
Figure 2-1. Cell proliferation in GM-CSF-treated cultured rat microglia as determined by MTT analysis. Following stimulation with the mitogen rrGM-CSF (CSF), microglia undergo a significant burst (p<0.001) in proliferation from day 2 to 9. However, when cultured in the continual presence of CSF, microglia undergo rapid telomere shortening (Flanary and Streit, 2004), and exhibit a significant decrease (p<0.001) in proliferation (when comparing day 9 to 41, day 16 to 41, or day 27 to 41). Importantly, proliferation in CSF-stimulated microglia eventually falls to levels below that evident in un-stimulated control microglia (i.e., on day 41). This suggests that microglia in vitro undergoing continual rapid division apparently “use up” most of their replicative potential within the first 14 days (as evidenced by the smaller burst in proliferation from day 16 to 27 (p>0.05) compared to day 2 to 9), and are no longer able to sustain this rate at later timepoints, at which point they likely succumb to replicative senescence.

Telomere Shortening Occurs in Cultured Rat Microglia

Both control and rrGM-CSF (GM-CSF)-stimulated microglia underwent telomere shortening while in culture (Fig. 2-2). In control microglia, both the longest and mean telomere length remained relatively unchanged from day 1 to 16 while the shortest telomeres decreased a moderate 6.3 kb. In contrast, GM-CSF-stimulated microglia exhibited dramatic telomere loss (10.8 kb) in the longest telomeres from day 1 to 16, with
minimal shortening (0.9 kb) occurring from day 16 to 32. Mean telomere length decreased only slightly over time. Conversely, the quantity of short telomeres greatly increased (9.1 kb) from day 1 to 16, with a small increase (2.1 kb) occurring from day 16 to 32 (Fig. 2-3). Thus, in stimulated microglia, the longest telomeres were allowed to shorten while short telomeres were lengthened. Additionally, the shortest telomeres are continually being lengthened over time, even immediately prior to senescence (around day 32). The quantity of longest telomeres was much larger (9 kb) in GM-CSF-treated microglia than in controls on day 1 (Fig. 2-3). This indicates that rapid telomere lengthening occurs in microglia during the first 24 hours of being exposed to GM-CSF, and is supported by TRAP data which shows that telomerase activity is substantially higher in stimulated microglia, compared to unstimulated microglia. No DNA was available to be isolated on day 32 in control cells, and very little in GM-CSF-treated cells, since they were already senescent at this time point. When microglia were plated in various sized culture dishes and grown to confluence, telomere length diminished proportional to the size of the culture dish. With increasing culturing area (i.e., 9.5 cm², 21 cm², and 175 cm²), telomere length decreased in the longest (1.4 kb), mean (1.3 kb), and shortest (2.1 kb) telomeres (Fig. 2-4, 2-5).
Figure 2-2. Southern blot analysis for measurement of telomere length in cultured microglia. Genomic DNA was isolated on the indicated days (1, 16, 32) in cells treated with either sterile water (CON), or 10 nM rrGM-CSF (CSF). For both treatments, telomere length decreased over time. However, telomere shortening was much more pronounced in GM-CSF-treated cells.
Figure 2-3. Telomere length distribution in control and GM-CSF-stimulated microglia on days 1, 16, and 32.
Figure 2-4. Southern blot analysis for measurement of telomere length in microglia cultured at varying densities. Cells were plated without GM-CSF at varying densities and culture areas as follows: 5x10^5 cells (9.5 cm^2), 5x10^5 cells (21 cm^2), or 2x10^6 cells (175 cm^2). Genomic DNA was isolated once cells reached near-confluence following 3 days of growth. Increased telomere attrition is evident in microglia plated in larger culture areas evidently caused by their increased division, which was necessary in order to fill the available culturing area.
Three-Fold Variation Exists in Individual Rat Microglia Telomeres

Individual microglia telomeres were visualized using FISH analysis (Fig. 2-6). Telomere fluorescence intensity (TFI) is a measurement (arbitrary units) used to provide an indicator of the number of telomeric repeats present on a particular telomere. Microglia on day 2 of in vitro growth were found to have a normal karyotype consisting of 42 chromosomes. The TFI was found to vary greatly both between and within individual chromosomes (Fig. 2-7). Variation in TFI values on all chromosomes ranged from 17.0 to 52.0, representing a 3.1-fold maximal difference in telomere length (mean TFI = 44.2 ± 5.2). Variation of TFI values on the same chromosome ranged from 17.6 to 47.0, representing a 2.7-fold difference in telomere length.
Figure 2-6. Telomere FISH analysis of metaphase spreads of cultured rat microglia using a FITC-conjugated peptide nucleic acid telomere-specific probe. Cells were viewed at 90X (left) and 270X (right) magnification via confocal microscopy. Note the green telomeres at the ends of red chromosomes. Two large red cell nuclei containing interphase chromosomes are visible in the upper portion of the 90X view. Substantial telomere length variation was found to exist both between and within individual microglial telomeres. Scale bar is approximately 10 μm (in 90X view) and 5 μm (in 270X view).
Figure 2-7. Telomere fluorescence intensity (TFI) of all 168 individual telomeres in the 42 chromosomes of 2-day old cultures of rat microglia. TFI is an arbitrary measurement corresponding to the number of telomeric repeats present on each telomere. Thus, TFI is directly proportional to telomere length. Note the large quantity of long telomeres, as evidenced by high TFI values, which are present in young microglial cultures.

**Cyclical Telomere Shortening Occurs in Rat Astrocytes**

Astrocytes were also found to undergo telomere shortening during the first 10 days of *in vitro* culturing (Fig. 2-8). From day 1 to 3, telomere length decreased rapidly, with the differences being 12.7 kb in the longest, 6.1 kb in the mean, and 13.8 kb in the shortest telomeres. Beginning on day 3, a fluctuating pattern in telomere length in all groups (i.e., longest, mean, shortest) begins to develop. From day 3 to 4, telomeres in all
groups (except the longest) are lengthened, most notably the shortest (10 kb). However, from day 4 to 5, telomeres in all groups shorten. From day 5 to 7, telomeres in all groups are again lengthened. Then, from day 7 to 10, telomeres in all groups (except the longest from day 8 to 9) shorten again (Fig. 2-9).

Figure 2-8. Southern blot analysis for measurement of telomere length in non-passaged astrocytes from day 1 to 10. Telomere length decreased over time, especially during days 1 to 3. A cyclical pattern of telomere shortening and re-lengthening was observed from day 1 to 10.
Figure 2-9. Telomere length distribution in non-passaged astrocytes from day 1 to 10. Note the cyclical pattern of telomere lengthening and shortening that occurs with time in culture.

- During lengthening, the shortest telomeres are lengthened the quickest, suggesting that maintenance of the shortest telomeres is most critical (Hemann et al., 2001).
- Astrocytes were also found to have consistently longer telomeres in all groups compared to microglia. Mean telomere length in astrocytes on day 1 is 6.1 kb longer than in GM-CSF-treated microglia, and 9.2 kb longer than in control microglia on the same day. The longest telomeres in astrocytes on day 1 are 7.7 kb longer than in GM-CSF-treated microglia, and 16.7 kb longer than in control microglia on the same day. Similarly, the shortest telomeres in astrocytes on day 1 are 10.6 kb longer than in control microglia, and 18.5 kb longer than in GM-CSF-treated cells on the same day (Fig. 2-3 and 2-9). Long-
term growth of cultured astrocytes (up to day 32) revealed a similar pattern of telomere lengthening followed by a period of telomere erosion (Fig. 2-10). From day 2 to 16, telomere length increased in the longest (4.9 kb), mean (4.1 kb), and shortest (1.1 kb) telomeres. Similarly, from day 16 to 32, telomeres shorten in the longest (3.5 kb), mean (3.0 kb), and shortest (1.2 kb) telomeres (Fig. 2-11). This cyclical pattern of telomere lengthening and shortening in astrocytes is supportive of a similar cyclical pattern of transient telomerase expression and repression that is occurring in these cells.

Figure 2-10. Southern blot analysis for measurement of telomere length in non-passaged rat astrocytes from day 2 to 32. Telomere length increased from day 2 to 16, then declined from day 16 to 32. This cyclical pattern of telomere lengthening and attrition correlates to increasing and decreasing telomerase activity over the same time period.
Figure 2-11. Telomere length distribution in non-passaged rat astrocytes from day 2 to 32. Note the cyclical pattern of telomere shortening and lengthening that occurs with time in culture.

When astrocytes are passaged serially, mean telomere length remains nearly unchanged from passage 1 to 5 (Fig. 2-12). However, both the longest and shortest telomeres undergo a cyclical pattern of telomere lengthening and shortening. Both sets of telomeres lengthen from passage 1 to 2, and then shorten from passage 2 to 3. Subsequently, they re-lengthen from passage 3 to 4, and then shorten from passage 4 to 5. The shortest telomeres exhibit the most dramatic lengthening. From passage 1 to 2 they lengthen 7.4 kb, and from passage 3 to 4 they lengthen 9.7 kb. The overall trend for the shortest telomeres is a continual lengthening over time (Fig. 2-13).
Figure 2-12. Southern blot analysis for measurement of telomere length in astrocytes from passage 1 to 5. The interval between each passage was as follows: passage 1 to 2 (4 days), 2 to 3 (6 days), 3 to 4 (6 days), 4 to 5 (9 days). While the mean telomeres remain nearly unchanged, the longest and shortest telomeres undergo a cyclical pattern of telomere lengthening and shortening, with the shortest telomeres gradually lengthening over time.
Figure 2-13. Telomere length distribution in astrocytes from passages 1 to 5. Note the cyclical pattern of telomere shortening and lengthening that occurs with time in culture.

**Telomerase Activity in Cultured Rat Microglia and Astrocytes**

Telomerase activity was measured in both microglia and astrocytes on various days (Fig. 2-14 to 2-16). Telomerase activity was consistent, and at a low level, in control microglia from day 0 to 32. In contrast, GM-CSF-stimulated microglia express a nearly 3-fold increase in telomerase activity on day 2, compared to controls. Subsequently, telomerase activity follows a cyclical pattern and declines until day 24, then increases dramatically again from day 24 to 32 (Fig. 2-14 and 2-16). In astrocytes, telomerase activity gradually increased from day 0 to 2, then rapidly increased from day 2 to 16. Telomerase activity then dramatically declined from day 16 to 24. By day 32, activity was nearly non-existent (Fig. 2-15, 2-16). Telomerase activity correlated well
with telomere length in astrocytes. Mean telomere length was found to increase in astrocytes by 4.1 kb from day 2 to 16, corresponding to a period of high telomerase activity, especially on days 9 to 16 (during which time telomeres were lengthened). Subsequently, mean telomere length decreased 3.0 kb from day 16 to 32, corresponding with a period of low/absent telomerase activity (during which telomeres shortened) (Fig. 2-11 and 2-16).

Figure 2-14. Telomerase activity in control (Con) and GM-CSF (CSF)-stimulated rat microglia on the indicated days. Telomerase activity in control microglia remains relatively consistent. However, in GM-CSF-stimulated microglia, telomerase activity is increased dramatically on days 2 and 32, compared to controls. (-) = telomerase-negative control; (+) = telomerase-positive control; IC = internal control.
Figure 2-15. Telomerase activity in non-passaged rat astrocytes on the indicated days.
Telomerase activity in astrocytes exhibits a cyclical pattern of increasing and decreasing telomerase activity. (-) = telomerase-negative control; (+) = telomerase-positive control; IC = internal control.
Figure 2-16. Quantitation of telomerase activity (arbitrary units) in rat microglia and non-passaged astrocytes on the indicated days. Control microglia express low telomerase activity than the positive control. GM-CSF-stimulated microglia exhibit a cyclical cycle of increasing and decreasing telomerase activity. Telomerase activity in astrocytes on day 0 is nearly 3-fold higher than in microglia on the same day and was always higher than the positive control. Additionally, GM-CSF-stimulated microglia exhibit a 3-fold higher level of telomerase activity on day 2, compared to controls.

**Discussion**

We have shown that both microglia and astrocytes undergo dynamic changes in both telomere length and telomerase activity with time in culture. While telomere shortening occurs gradually in actively dividing microglia and is accompanied by their progression into senescence, astrocytes exhibit a cyclical pattern of telomere lengthening and shortening and are able to divide for much longer periods of time *in vitro* than microglia. Our study, which is the first to report on telomere length and telomerase
activity in glial cells of any organism, shows that telomeres shorten progressively in microglia with increased cell division and with time in culture.

The fact that microglia can be induced to proliferate following CNS injury suggests that microglia represent a self-renewing population of cells. Our current findings open the possibility that the replicative potential of microglia in vivo is limited and that these cells may at some point exhaust their replicative capacity. Thus, injury-induced mitosis of microglia in vivo could result in telomere shortening, which may drive the cells down an accelerated path towards cellular senescence.

Rapid elongation of the shortest telomeres in GM-CSF-treated cells occurs from day 1 to 16, suggesting that these cells are trying to evade senescence by maintaining the shortest telomeres above the critical length. The shortest telomeres lengths in GM-CSF-treated cells are 14.4 kb on day 1, while controls have lengths of 16 kb on day 16 (and enter senescence shortly thereafter). Thus, the critical telomere length that triggers senescence appears to be somewhere slightly below 14.4 kb. Control microglia exhibit telomere attrition of the shortest telomeres, while the longest and mean telomeres remain nearly unchanged, from day 1 to 16, which suggests that either the longest telomeres are being preferentially maintained, or that non-uniform telomere lengthening occurs such that the longest telomeres actually increase in size (by 0.5 kb) while the shortest continue to erode (by 6.3 kb). The data also suggests that attrition of the shortest telomeres occurs in the presence of low levels of telomerase (since both longest and mean telomeres are maintained). This is supported by our TRAP data indicating that telomerase is present in low amounts, which is likely enough to maintain the longest and mean telomeres, while the shortest telomeres continually erode. Unstimulated cells appear to enter senescence
when telomeres reach critical lengths, however, the GM-CSF-stimulated microglia do not. No critical length is reached in these stimulated cells, yet they still undergo senescence, as evidenced by reduced mitotic activity and viability after several weeks in culture, suggesting that mechanisms other than critically-short telomeres (e.g., ROS damage, cellular trauma, shortening of the longest telomeres) may be triggering senescence in these cells in the absence of telomerase repression (Kodama et al., 2001).

Telomere erosion is also thought to occur directly by other means, which are independent of cell replication (von Zglinicki, 2002). Since GM-CSF-treated microglia on day 1 have longest telomeres that are 9 kb longer than in controls on the same day, GM-CSF may up-regulate high telomerase activity during the first few days of growth (Szyper-Kravitz et al., 2003), which is likely to prepare the cells for the rapid division that subsequently occurs. This idea is supported by our TRAP data, which shows greatly increased telomerase activity in microglia from day 0 to 2 (i.e., nearly 3-fold higher, compared to controls). Following day 1, GM-CSF treatment induces only maintenance of the shortest telomeres, as indicated by their continual lengthening until senescence, while the longest and mean telomeres are allowed to shorten. After day 2, telomerase activity in GM-CSF-stimulated microglia steadily declines until day 24, then increases dramatically again to day 32. This supports our Southern blot data, indicating that telomeres continue to be lengthened until senescence. The elongation of short telomeres (by 9.1 kb) in GM-CSF-stimulated microglia from day 1 to 16 corresponds with a nearly 3-fold higher increase in telomerase activity during this time. Similarly, short telomeres increase by 3.1 kb from day 16 to 32, corresponding to an approximate 2-fold increase in telomerase activity during this time. It is apparent that the rate of elongation of short
telomeres (relative to measured telomerase activity) from day 16 to 32 is lower compared to day 1 to 16. At day 32, nearly all microglia are likely senescent, and thus may not be able to lengthen telomeres as efficiently or recruit telomerase as proficiently as on day 2, suggesting that microglia are less able to utilize available telomerase with increasing time in culture. The level of telomerase activity is likely enough to maintain the shortest telomeres while the longest and mean telomeres continually erode. Undergoing rapid division, as during GM-CSF treatment, appears to enable microglia to rapidly increase telomere length initially, then on subsequent days to recruit telomerase to the shortest telomeres, while allowing the longest and mean telomeres to shorten. The opposite is seen in control microglia, in which only the shortest telomeres erode. Thus, a mechanism may exist in microglia (following periods of rapid division) that preferentially recruits limiting amounts of telomerase to maintaining the shortest telomeres while allowing the longer telomeres to shorten (Ouellette et al., 2000). Telomerase activity may seem apparent when total protein is isolated and measured following in vitro culturing, yet the enzyme could be inhibited by a repressor molecule while in vitro or in vivo, which may or may not be present within the total protein pool during analysis. Additional in vitro or in vivo molecules may also play critical roles in regulating telomerase activity. Thus, the pattern of telomerase activity, as determined by in vitro total protein analysis, may not correlate precisely with or imply telomere maintenance in vitro or in vivo (Ouellette et al., 1999).

Control microglia are already senescent before day 32 while GM-CSF-stimulated cells are at or near senescence on the same day, suggesting that GM-CSF treatment may result in a slightly increased cell life span due to the absence of critically-short telomeres.
Our results suggest that the slightly increased life span of GM-CSF-treated microglia may be due to their enhanced ability to maintain the shortest telomeres (by telomerase recruitment), which may result in their delayed entry into senescence, compared to controls. Perhaps during periods of rapid division, microglia are able to more proficiently recruit telomerase to the shortest telomeres in an attempt to enable cell division to occur for a longer period of time prior to entry into senescence. Therefore, the replicative capacity of rapidly-dividing microglia appears to be greater compared to that of controls. Microglia were considered senescent by day 32 in control and GM-CSF-treated groups since they both exhibited decreased proliferation, telomere shortening, and altered phenotypes (relative to non-senescent dividing microglia). Despite continuous GM-CSF-stimulation, microglia were unable to maintain a high cell division rate, and most of the cells had sloughed off from the culture dish by day 32, indicating that they were no longer viable. Indeed, the yield of DNA was much lower, such that the entire DNA sample was used for Southern blot analysis for GM-CSF-treated cells on day 32, whereas only a fraction of the total DNA collected was used for analysis in all other days. Microglia grown to near-confluence in various-sized culture dishes exhibit increased telomere attrition with increasing available culture area. Telomere loss likely occurs in these cultures due to additional cell divisions in microglia that are necessary to reach near-confluence in the larger-sized culture dishes. There were 4 times as many microglia plated in the 175 cm$^2$ flask compared to the 9.5 and 21 cm$^2$ dishes. However, the 175 cm$^2$ flask had a culture area that was over 18 times greater than the 9.5 cm$^2$ dish and over 8 times greater than the 21 cm$^2$ dish. Thus, it took approximately 4.5 more divisions per cell to reach near-confluence in the 175 cm$^2$ flask compared to the 9.5 cm$^2$ flask. These
additional divisions likely account for the decrease in the shortest telomeres evident in microglia cultured in the 175 cm² flask compared to the other smaller-sized culture dishes. Importantly, the shortest telomeres in microglia cultured in the 175 cm² flask are up to 3.2 kb shorter, representing an 8.4% decrease, than those found in microglia cultured in the two smaller-sized dishes. These data demonstrate that the rate of erosion of the shortest telomeres in microglia is directly proportional to the number of divisions that a cell undergoes.

Analysis of microglia telomeres by FISH revealed that substantial telomere length variation occurs both between and within individual chromosomes. This indicates that a heterogeneous population of telomeres exist within each microglial cell and among sister chromatids (Bekaert et al., 2002), and further suggests that only a few individual chromosomes likely reach a critical length first (thereby triggering cell senescence), assuming uniform telomere shortening with age. The telomeres that are the first to reach a critical length are likely the first to induce SAGE and TPE on these chromosomes. Thus, genes (especially those nearest the telomeres) may be up-regulated with age in microglia due to TPE (Wright and Shay, 1992). Why does so much telomere length variation exist, especially within the same chromosome? Telomeres on individual chromosomes may be longer than those on other chromosomes in order to keep gene regulation and expression constant for that particular area of the chromosome (especially genes nearest the telomere), or to prevent SAGE and TPE from occurring when telomeres shorten sufficiently. It would be interesting to determine which genes are located nearest the telomere on chromosomes which reach a critical length first, since this may provide clues as to the genetic changes that occur in microglia as they age. Furthermore, this may
suggest an approach (e.g., telomerase over-expression) that would prevent TPE-induced expression of detrimental genes on chromosomes possessing very short telomeres. Future studies will permit quantitative FISH (Q-FISH) telomere length measurements, in which telomere lengths will be quantitated, compared to a standard curve of fluorescence intensity of plasmids containing known telomeric repeats (Poon et al., 1999). Since sub-telomeric regions will not be bound by probe (as is the case for Southern blot), this will allow a more precise measurement of telomere length. Q-FISH will allow a determination of how much inter- and intra-chromosomal telomere length variation occurs in microglia and astrocytes with age, will help identify which chromosomes are the first to reach a critical length and trigger senescence, and if certain chromosomes are more susceptible to telomere attrition.

Telomeres in astrocytes shorten dramatically during the first few days of in vitro growth, which is consistent with their rapid division during this time to reach confluence. A fluctuating pattern of telomere maintenance and attrition follows, which is accompanied by transient telomerase up-regulation during periods of telomere maintenance. This fluctuating cycle continues as the cells are maintained through day 32 without passaging, but it is unknown how long this cycle would continue. In astrocytes, senescence does not occur during these 32 days in culture. This shows that, at least in vitro, astrocytes have a longer life span than microglia. Perhaps this is because astrocytes have longer telomeres overall than microglia, and/or because telomerase may be inhibited in astrocytes while in vivo, but have high activity (due to lack of inhibition) when these cells are cultured. Small variations exist in telomere lengths between figures 5 and 6, both of which examine non-passaged astrocytes. This can be attributed to the numerous
experimental parameters that can contribute to the slight variability present from one experiment to another due to the following: different exposure times (ranging from 1 to 2 hours) during development of the Southern blots, different hybridization times (ranging from 17 to 19 hours) that the membrane is in contact with the telomere probe, and especially since different cultures of cells are used for each experiment (each culture has cells with different rates of mitosis and varying concentrations (about 1.5 rat pup brains per 175cm$^2$ flask) of whole brain cultures plated initially, which would lead to different division rates of both astrocytes and microglia prior to their collection, and could therefore account for this variability).

In passaged astrocytes, mean telomere length remains nearly constant while the longest, and especially the shortest, telomeres fluctuate over time. This suggests that transient expression of low levels of telomerase may be occurring such that telomerase is preferentially recruited to the shortest telomeres, while mean telomere length remains unchanged (Ouellette et al., 2000). Thus, telomerase may be up-regulated (and therefore maintaining telomere length) during periods of rapid division, as when astrocytes are first passaged. Cultured astrocytes replicate continuously when passaged, compared to when they are plated in 9.5 cm$^2$ dishes and not passaged. However, it was noted that with continual passaging, a slowing of cell division occurred such that it took an increasingly longer amount of time to reach confluence with successive passages. Once astrocytes reach confluence in the 9.5 cm$^2$ dish, little replication is likely to occur thereafter. Thus, non-passaged astrocytes undergo aging in the absence of cell division. However, when astrocytes are permitted to replicate continually (as when passaged), they maintain telomeres and even lengthen the shortest ones over time. As in GM-CSF-stimulated
microglia, telomeres are initially lengthened when considerable replication is about to occur. Thus, given the opportunity to continuously replicate, perhaps both microglia (when exposed to GM-CSF) and astrocytes (when passaged) up-regulate telomerase to compensate for telomere shortening that would otherwise occur. Future studies will examine telomere length and telomerase activity in microglia and astrocytes \textit{in vivo}.
Chapter 3
Telomeres Shorten with Age in Rat Cerebellum and Cortex in Vivo

Introduction

Recent data from our laboratory (Flanary and Streit, 2004) has shown that telomeres shorten with time in cultured microglia, suggesting that these brain cells are subject to replicative senescence in vivo. Thus, we decided to determine how telomere length and telomerase activity change in the rat brain with aging. Our results show that telomere erosion occurs in vivo in the rat cerebellum and cortex with age in the presence of low levels of steadily increasing telomerase activity.

Materials and Methods

Collection of Rat Cerebellum and Cortex Tissues

Sprague-Dawley rats were maintained at 22°C in a controlled 12 hour light/dark cycle and provided food and water ad libitum. Animals were euthanized by exsanguination using transcardiac perfusion with phosphate-buffered saline under deep anesthesia with sodium pentobarbital (50 mg/kg body weight). This method of euthanasia is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Following perfusion, the cerebellum and cortex were dissected out and frozen prior to DNA and protein isolation.

Determination of Telomere Length

Telomere length was measured as described in Chapter 1.
Determination of Telomerase Activity

Telomerase activity was measured as described in Chapter 1.

Results

Telomeres Shorten With Age in Rat Brain \textit{in vivo}

Both rat cerebellum and cortex tissue exhibit telomere shortening \textit{in vivo} from day 21 to 152 (Fig. 3-1, 3-2). The cortex always had shorter telomeres (i.e., longest, mean, and shortest) than the cerebellum, except on day 152 in the longest telomeres (Fig. 3-3). In cerebellum, the longest telomeres shortened the most rapidly (loss of 5.6 kb, or 26%), followed by the mean telomeres (loss of 2.3 kb, or 17%), and the shortest telomeres (loss of 1.6 kb, or 18%) from day 21 to 152. In the cortex, the mean telomeres shortened most rapidly (loss of 2.9 kb, or 22%), followed by the longest telomeres (loss of 2.2 kb, or 12%), and the shortest telomeres (loss of 1.0 kb, or 13%) from day 21 to 152. In both cerebellum and cortex tissues, the shortest telomeres were the slowest to shorten with age \textit{in vivo} (Fig. 3-3).

Telomerase Activity in Rat Cerebellum and Cortex

Telomerase activity was measured in both cerebellum and cortex on various days. With increasing age \textit{in vivo}, telomerase activity steadily increased in both tissues from day 21 to 182, with the cerebellum exhibiting the highest activity at all time points examined (Fig. 3-4, 3-5). From day 21 to 182, telomerase activity increased 28% in the cerebellum and 11% in the cortex. Further analysis of telomerase activity in cerebellum and cortex from day 21 to 35 revealed that activity increased slightly in all samples with age \textit{in vivo} except for one cerebellum sample from day 28 to 35 (Fig. 3-6, 3-7). Overall, telomerase activity increased with age \textit{in vivo} from day 21 to 35 in both cerebellum and cortex, with the cerebellum exhibiting the highest activity at all time points measured.
From day 21 to 35, telomerase activity increased 12% in the cerebellum and 11% in the cortex (Fig. 3-8).

Figure 3-1. Southern blot analysis for measurement of telomere restriction fragment (TRF) length in rat brain tissue. Genomic DNA was isolated on the indicated days (day 152 is approximately 5 months of age) from cerebellum and cortex tissues of two different rats (A and B).
Figure 3-2. TRF length distribution in rat cerebellum and cortex samples on days 21 and 152.

Figure 3-3. Average TRF length in rat cerebellum and cortex tissues on days 21 and 152. Overall, the cerebellum has longer telomeres than the cortex from day 21 to 152 in all instances (except in longest telomeres on day 152). Telomere shortening occurs with age *in vivo* in both rat cerebellum and cortex. Although only two animals were analyzed for each time point (except cortex day 152), no overlap of error bars exists between cerebellum and cortex in any instances.
Figure 3-4. TRAP analysis for telomerase activity in rat brain tissue (days 21 to 182).

Total protein was isolated on the indicated days (days 152 and 182 are approximately 5 and 6 months of age, respectively) from cerebellum and cortex tissues. Neg = telomerase-negative control (i.e., cortex A day 21 RNAse-treated extract). Pos = telomerase-positive control.
Figure 3-5. Quantitation of telomerase activity (arbitrary units) in rat cerebellum and cortex tissues (days 21 to 182). Telomerase activity gradually increases with age \textit{in vivo} in both cerebellum and cortex in all instances.

Figure 3-6. TRAP analysis for telomerase activity in rat brain tissue (days 21 to 35). Total protein was isolated on the indicated days from cerebellum and cortex tissues of two different rats (B and C).
Figure 3-7. Quantitation of telomerase activity (arbitrary units) in rat cerebellum and cortex tissues (days 21 to 35). Telomerase activity gradually increases with age in vivo in both cerebellum and cortex in all instances (except cerebellum B from day 28 to 35).

Figure 3-8. Overall, the cerebellum exhibits higher telomerase activity than the cortex from day 21 to 35.
Discussion

This study, which is the first to report on both telomere length and telomerase activity in a region-specific manner in rat brain, shows that telomere shortening occurs in both rat cerebellum and cortex with increasing age in vivo. The telomere shortening is accompanied by low levels of steadily increasing telomerase activity, which is highest in the cerebellum in all instances.

Our current findings on telomere length in brain tissues are in apparent conflict with a recently published study (Cherif et al., 2003). These authors report that while telomeres shorten with age in rat kidney, liver, lung, and pancreas, no telomere shortening occurs in rat brain tissue with age (from postnatal day 21 to 15 months of age). However, their data does indicate that both the longest and shortest telomeres do shorten in rat brain with age, but not significantly. In addition, the analyses in the study (Cherif et al., 2003) were based on unspecified brain areas, while our current findings are in defined brain regions and showed corresponding results (i.e., telomeres shorten in rat brain with age). Moreover, it appears that the animals used in the study (Cherif et al., 2003) were not perfused prior to tissue collection, and thus it is likely that the brain tissue used for telomere length analysis represented a mixture of brain and blood tissue. Blood contamination of brain tissue could have obscured any brain-specific telomere shortening. On the other hand, there are two independent studies which have reported that telomeres do undergo shortening in brain tissue with age. One study performed in mice demonstrated that telomere shortening does occur in spleen and brain tissue, but not in liver, testes, or kidney with age from zero to nine months (Prowse and Greider, 1995). Another study also found that telomeres shorten in mice brain tissue with age from one to 24 months (Coviello-McLaughlin and Prowse, 1997). These three studies (Cherif et al.,
2003; Prowse and Greider et al., 1995; Coviello-McLaughlin and Prowse, 1997) are the only ones known, at the time of this writing, that have examined telomere length in brain tissue of rodents with age. Clearly, more work will be required to resolve any conflicting results that may exist now and to reach a satisfactory conclusion about telomere erosion in the CNS.

The rate of telomere loss of the longest telomeres in the cerebellum from day 21 to 152 is over two times greater than that in the cortex. This suggests that more cell division occurs in the cerebellum during this time frame in vivo, compared to the cortex. Our current study has revealed a difference in telomere lengths between the cerebellum and cortex by showing that the longest, mean, and the shortest telomeres in the cortex are consistently shorter than those found in the cerebellum in all but one instance. This observation may, at first, seem unexpected since cerebellar, unlike cortical, histogenesis is characterized by the existence of a secondary proliferative zone during late stages of cerebellar development (Steward, 2000), and one might therefore expect to see shorter telomeres in the cerebellum. However, our data also show higher telomerase activity in the cerebellum, compared to the cortex, which thereby may account for the presence of longer telomeres (despite cell division in the secondary proliferative zone) in the cerebellum compared to the cortex. Few studies have examined telomerase activity in the brain. One study (Klapper et al., 2001) reported that telomerase activity is high in mouse cortex during embryonic development, but sharply decreases during postnatal development up to three months of age. Likewise, another study (Fu et al., 2000) reported that telomerase activity is high in rodent neurons during embryonic and early postnatal development, but then subsequently decreases. Telomerase activity is also high
in rat oligodendrocyte precursor cells, but declines during their differentiation into oligodendrocytes (Caporaso and Chao, 2001). Our current results indicate that telomerase activity is lowest on day 21 and steadily increases, albeit slightly, up to six months of age. In comparison to previous research in this area (Klapper et al., 2001), our results may or may not coincide. The slight increase in telomerase activity reported in our study from day 21 to 6 months of age may similar to the same low/decreased levels as those found in previous studies (Klapper et al., 2001) (after early postnatal development) if sample sets from both studies were compared on the same gel. It is nearly impossible to make an accurate comparison between different TRAP gels (especially between different laboratories), since many factors (e.g., annealing temperature, number of PCR cycles, protein and primer concentration used, etc.) influence ladder formation, band intensity, and subsequent telomerase activity levels (which are expressed in arbitrary units, or as a percentage of the highest levels evident).

The longest telomeres (in cerebellum) and the mean telomeres (in cortex) undergo the highest rate of telomere loss with age \textit{in vivo}. However, in addition to the longest telomeres in the cortex, the shortest telomeres in both tissue types exhibit the slowest rate of attrition with age. Since telomerase activity slightly increases in both tissue types with age, this suggests that limiting amounts of telomerase may be preferentially recruited to the shortest telomeres, while allowing the longest telomeres to shorten (Ouellette et al., 2000). This data also suggests that the amount of telomerase present may not be enough to sufficiently compensate for the rate of telomere loss that occurs in the shortest telomeres with age, since telomeres in both tissue types still shorten with age.
There is high mitotic activity of different, primarily glial, cell types in the developing rodent brain during the neonatal/postnatal period up to about postnatal day 14. On postnatal day 21, most mitotic activity has ended and the CNS is almost fully matured. At this time, microglia are probably the only adult cell type remaining in the postnatal CNS that undergo any appreciable cell division, and they retain their mitotic ability throughout adult life. Since our analyses began on day 21, we are inclined to think that most of the telomere erosion subsequent to this day that occurs in both cerebellum and cortex may be largely attributable to cell division of microglia. Of course, we cannot exclude the possibility that some of the observed telomere attrition may also be contributed by neural stem cells in the subventricular zones. Future studies employing tissue from the hippocampus could further illuminate this issue, since neurogenesis occurs in the hippocampus throughout adult life, but declines with aging (Kuhn et al., 1996). In addition, some of the observed telomere attrition could also be attributable to mechanisms not related to cell division, such as oxidative stress (von Zglinicki, 2002), in postmitotic cells. Future studies in our laboratory will examine telomere length and telomerase activity in vivo in rat cerebellum and cortex over a longer time period (up to several years) as well as in acutely isolated microglial cells from young and aged adult rats.
CHAPTER 4
AXOTOMY INCREASES TELOMERE LENGTH, TELOMERASE ACTIVITY AND PROTEIN IN AXOTOMY-ACTIVATED MICROGLIA

Introduction

Previous research in our laboratory has demonstrated that telomere shortening and senescence occurs in cultured rat microglia following periods of prolonged and sustained mitotic activity induced by GM-CSF (Flanary and Streit, 2004). In contrast, microglia in vivo undergo short proliferative bursts soon after an acute injury has occurred. This, together with the fact that microglia produce growth factors and cytokines after injury, suggests that mitosis affords a mechanism to provide greater numbers of microglial cells and thus greater trophic support during times of stress (Streit et al., 2000; Streit, 2002a). However, the mitotic potential of microglia also suggests that these cells have limited cellular life-spans and thus may rely on proliferation and self-renewal to replace senescent cells. In order to determine whether neuronal injury-induced microglial proliferation within a well-defined region of the CNS results in microglial telomere shortening or cellular senescence, we decided to measure parameters indicative of telomeric maintenance, such as telomere length and telomerase activity.

Materials and Methods

Rat Facial Nerve Axotomy

Adult Sprague-Dawley rats of both genders were housed at 22°C in a controlled 12 hour light/dark cycle and provided food and water ad libitum. Animals were anesthetized using isofluorane, and the right facial nerve was exposed and transected at the
stylomastoid foramen. Failure to move whiskers on the right side of the face following
recovery from anesthesia was used to verify the success of the axotomy. Animals were
euthanized at 1, 4, 5, 7, and 10 days post-axotomy by exsanguination using transcardiac
perfusion with phosphate-buffered saline (PBS) under deep anesthesia with sodium
pentobarbital (50 mg/kg body weight). This method of euthanasia is consistent with the
recommendations of the Panel on Euthanasia of the American Veterinary Medical
Association. Following perfusion, the entire brain was removed, snap-frozen 2-
methylbutane cooled by liquid nitrogen, and stored at -80°C. Individual facial nuclei of
both axotomized (i.e., right) and control (i.e., left) sides were micro-dissected using a
cryostat and stored at -80°C prior to DNA and protein isolation.

FACS-Isolation of Rat Microglia from Micro-dissected Facial Nuclei

Animals were exsanguinated under deep sodium pentobarbital anesthesia (50
mg/kg body weight) using ice-cold PBS. The brains were removed and placed in PBS on
ice. Micro-dissection of facial nuclei from the chilled brains was performed immediately
after removal. Nuclei (approximately 7 mg wet weight each) from both axotomized and
non-axotomized sides were collected from six animals at 5 days post-axotomy. Six
nuclei from each side were pooled and processed according to established isolation
protocols (Carson et al., 1998). Fluorescence-activated cell sorting (FACS) analysis was
performed using a FACSVantage SE cell sorter and CellQuest software (BD
Biosciences/Becton Dickinson, San Jose, CA). Monoclonal antibodies used to isolate
microglia during FACS analysis were FITC-conjugated anti-rat CD45 (leukocyte
common antigen), and PE-conjugated anti-rat CD11b/c (CR3 complement) (BD
Biosciences/Pharmingen, San Diego, CA), with microglia being identified as the CD11b/c high and CD45 low cell population (Ford et al., 1995; Sedgwick et al., 1991).

**Determination of Telomere Length**

Telomere length was measured as described in Chapter 1.

**Determination of Telomerase Activity**

Telomerase activity was measured as described in Chapter 1.

**Telomerase Western Blot Analysis**

Telomerase protein quantity was measured using Western blot analysis and chemiluminescent detection. Total protein was isolated as described above, and 50 μg from each sample, and a telomerase-positive control (rat glioblastoma cell line RG-2), was separated on an 8% SDS-PAGE gel at 25 mA for 1 hour. Protein was transferred from the gel to an Immobilon PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA) using semi-dry transfer at 5 volts for 1 hour. The membrane was blocked in 5% milk solution in TBST (tris-buffered saline with 0.1% Tween-20) for one hour at room temperature with shaking, and then incubated overnight in a primary antibody (rabbit polyclonal anti-telomerase antibody: EST-21A) (Alpha Diagnostics, San Antonio, TX) at a 1:250 dilution (4 μg/mL) in 5% milk solution in TBST at 4°C with gentle agitation. A second primary antibody (rabbit polyclonal anti-telomerase antibody: NB 100-141) (Novus Biologicals, Littleton, CO) was also used in parallel experiments with the Alpha Diagnostics antibody. Both antibodies yielded similar banding patterns, however, we found that the Novus Biologicals antibody gave very high background, and thus used the antibody from Alpha Diagnostics. Following washes in TBST for 4 x 15 min. at room temperature with fast shaking (120 rpm), a secondary antibody (horseradish peroxidase (HRP)-conjugated anti-rabbit) was applied for one hour at room temperature
with gentle agitation. Following washes in TBST for 4 x 15 min. at room temperature with fast shaking (120 rpm), chemiluminescent detection was accomplished by incubating the membrane in the HRP-metabolizing substrate ECL (enhanced chemiluminescence) (Amersham Biosciences, Piscataway, NJ) for one minute at room temperature. Following exposure of the membrane to X-omat AR Film (Eastman Kodak Company, Rochester, NY) in an autoradiography cassette for 1 to 10 min., the film was developed using a Konica SRX-101A automatic film processor (Konica Minolta, Mahwah, NJ). A digital image of the autoradiograph was generated by scanning it using a GS-710 calibrated imaging densitometer (BioRad, Hercules, CA). Quantitation of telomerase protein quantity was performed using the densitometry computer program Quantity One (version 4.3.1) (BioRad, Hercules, CA). Following development, the membrane was re-probed with an anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody as a loading control. The band corresponding to telomerase protein (catalytic portion) appeared at approximately 60 kDa, while GAPDH appeared at approximately 40 kDa. Normalized telomerase protein levels were determined by comparing the average densitometric values of identical samples ran on different gels in order to make an accurate comparison of protein levels between all samples run on multiple gels.

**Histochemistry**

Animals were perfused with 4% paraformaldehyde at 3 days post-axotomy. Dissected tissues were post-fixed for several days, then rinsed and stored in PBS. Following paraffin-embedding, serial sections (7.0 μm thick) were cut on a microtome (model 2040, Reichert-Jung, Buffalo, NY), mounted onto gelatin-coated slides, and
allowed to dry. Deparaffinization and rehydration were performed by soaking the slides in xylenes (2 x 15 min.), followed by passage through descending ethanols, including soaking in 70% ethanol overnight. Lectin histochemistry for the localization of microglial cells, using the *Griffonia simplicifolia* B4 isolecitin coupled to horseradish peroxidase (i.e., GSI B4-HRP) (catalog # L5391, Sigma-Aldrich, St. Louis, MO), was performed as described by Streit (1990). Following development with the peroxidase substrate diaminobenzadine (DAB), slides were coverslipped with Permount. Selected sections were counterstained with cresyl violet. Slides were photographed using a Zeiss Axioskop 2 plus microscope with a RT color Spot camera (model 2.2.1, Diagnostic Instruments, Inc., Sterling Heights, MI). Photographs were edited using Spot software (version 3.4.5) (Diagnostic Instruments, Inc., Sterling Heights, MI).

### Statistical Analysis of Data

Data was analyzed using the software program InStat version 3.06 (GraphPad, San Diego, CA). In order to determine whether a statistically significant difference existed between treatment groups, analysis of variance (ANOVA) was performed using the Tukey-Kramer multiple comparison test for TRAP assays, while an unpaired t-test was performed while for Western blot experiments. A p value of >0.05 was considered non-significant.

### Results

**Increase in Microglia Surrounding Axotomized Facial Nuclei**

Histological examination of sections confirmed prior descriptions of microglial activation in the axotomized facial nucleus (Fig. 4-1). On the axotomized side, increased numbers of glial nuclei were apparent and increased lectin staining confirmed that this was due to greater numbers of microglia. The activated microglia often were gathered
around axotomized motor neurons encircling them with their processes. Nuclear staining with cresyl violet revealed the presence of mitotic figures showing active microglial proliferation.

**Increase in Telomere Length in Axotomized Facial Nuclei**

Southern blotting was used to perform telomere length analysis in pooled micro-dissected rat facial nuclei (Fig. 4-2), with quantitation shown in Fig. 4-3. Telomere length in axotomized facial nuclei (4 days post-axotomy) increased in the longest (by 21%), mean (by 9%), and shortest (by 10%) telomeres, compared to the control facial nuclei. This indicates that telomeres lengthen in whole facial nuclei tissue following axotomy.
Figure 4-1. Micrographs of axotomized (A) and control (B) facial nucleus on day 3 post-axotomy stained with GSI-B4 lectin to identify microglia. Note the mitotic figure (black arrow, higher magnification shown in inset), and increased microglial cell numbers on the axotomized side, particularly around injured motor neurons (red arrows). Scale bar = 25 μm at 40X magnification (A and B), and 10 μm at 100X magnification (inset).
Figure 4-2. Southern blot analysis for measurement of telomere length in facial nuclei.
Figure 4-3. Densitometric quantitation of telomere length in facial nuclei. Genomic DNA was isolated from 10 pooled axotomized (A) and unlesioned control (C) facial nuclei on day 4 post-axotomy and probed with a digoxigenin-labelled telomere specific oligonucleotide (TTAGGG\text{3}). Densitometric quantitation revealed an increase in telomere length in axotomized facial nuclei.

**Increase in Telomerase Activity in Axotomized Facial Nuclei**

To test whether the observed increases in telomere length were attributable to similar increases in telomerase activity, TRAP analysis was used for determination of telomerase activity from day 1 to 10 post-axotomy in individual micro-dissected rat facial nuclei (Fig. 4-4). Normalized telomerase activity (i.e., of 3 separate TRAP gels with different samples normalized relative to each other) was significantly increased in the axotomized facial nuclei (compared to control nuclei) on days 1, 4, 7, and 10 by 8.96%, 68.74%, 104.05%, and 48.25%, respectively (Fig. 4-5). In a non-axotomized animal, no significant difference in telomerase activity existed between the right and left facial
nuclei (Fig. 4-6). These data suggest that one or more different cell types up-regulate telomerase activity following axotomy.

Figure 4-4. Representative TRAP analysis image used for measurement of telomerase activity in facial nuclei. Neg = telomerase-negative control; Pos = telomerase-positive control; IC = internal control.
Figure 4-5. Densitometric quantitation of telomerase activity in facial nuclei. Total protein was isolated from axotomized (A) or control (C) facial nuclei (day 4 post-axotomy, n = 4). Densitometric quantitation revealed an increase in mean (± SEM) normalized telomerase activity (arbitrary units) in axotomized facial nuclei at all timepoints, compared to controls (B). The percent increase in normalized telomerase activity in axotomized facial nuclei (compared to controls) following facial nerve axotomy increased the most from day 1 to 4, and peaked on approximately day 7. Day 0 represents a non-axotomized animal. Telomerase activity was significantly increased on day 4 (p<0.01), day 7 (p<0.001), and day 10 (p<0.05) compared to day 1, while day 7 was higher than day 10 (p<0.01). Total number of animals analyzed on each day was: day 1 (5), day 4 (6), day 7 (6), day 10 (5).
Figure 4-6. Densitometric quantitation of telomerase activity in unoperated facial nuclei. Absence of variation (p>0.05) in mean (± SD) telomerase activity (arbitrary units) between left and right non-axotomized facial nuclei from naïve, unoperated animals (C). Five independent samples were analyzed for both the left and right facial nuclei. Telomerase activity in the right facial nuclei was 1.42% higher than in left facial nuclei, but was not statistically significant.

**Increase in Telomerase Protein Quantity in Axotomized Facial Nuclei**

In order to determine if the increases evident in telomerase activity were due to an increased protein expression, Western blotting was used for determination of telomerase protein quantity in individual micro-dissected rat facial nuclei (Fig. 4-7). Quantitation of telomerase protein in rat facial nuclei is shown in Fig. 4-8. Normalized telomerase protein quantity (i.e., of 3 separate Western blot gels with different samples normalized relative to each other and to GAPDH) was higher in axotomized facial nuclei (compared to control nuclei) on day 1 (by 27.68%), 4 (by 45.13%), 7 (by 37.01%), and 10 (by
103.16%) post-axotomy. This suggests that one or more different cell types up-regulate telomerase protein following axotomy.

Figure 4-7. Western blot image used for measurement of telomerase protein quantity in facial nuclei. Total protein was isolated from axotomized (A) or control (C) facial nuclei on either 1, 4, 7, or 10 days post-axotomy (A). Pos = telomerase-positive control (RG-2 glioma cells).

Figure 4-8. Densitometric quantitation of telomerase protein in facial nuclei. An increase in mean (± SEM) normalized telomerase protein (arbitrary units) in axotomized facial nuclei at all timepoints was observed, compared to controls (B). The percent increase in normalized telomerase protein in axotomized facial nuclei (compared to controls) following facial nerve axotomy increased the most from day 1 to 4, and slowly declined from day 4 to 10. Telomerase protein was significantly increased (p<0.03) on day 4 compared to day 1 in the axotomized side; all other timepoints are non-significant (p>0.05). Axot = axotomized facial nuclei; Con = control facial nuclei.
FACS-Isolation of Microglia from Facial Nuclei

To determine if microglia were the cell type responsible for the increases in telomerase activity/protein (and hence telomere length), microglia were isolated from six pooled facial nuclei using CD11b/c and CD45 antibodies (Fig. 4-9). Since the samples contained mixed cell populations, cells were initially sorted based on the R1 gate, which excluded cellular debris (near the lower-left corner) and other undesirable cells (e.g., doublet and triplet cells) present elsewhere within the plot (other than R1). Microglia were identified as the CD11b/c high and CD45 low cell population (i.e., the R2 gate). The parameters of the R1 and R2 gates were determined in previous experiments (Carson et al., 1998). A total of 1,334 microglia (0.46% gated) were isolated from the pooled axotomized facial nuclei (Fig. 4-9H), whereas only 669 total microglia (0.22% gated) were isolated from the pooled control facial nuclei (Fig. 4-9J), indicating that at least twice as many microglia were present in the axotomized facial nucleus compared to the control nucleus.

Increase in Telomerase Activity in FACS-Isolated Microglia From Axotomized Facial Nuclei

TRAP analysis was used for determination of telomerase activity in FACS-isolated microglia from rat facial nuclei (Fig. 4-10). Quantitation of average telomerase activity in FACS-isolated microglia is shown in Fig. 4-11. Telomerase activity increased by 254.71% in FACS-isolated microglia from axotomized facial nuclei compared to control facial nuclei. This indicates that microglia within the axotomized facial nucleus exhibit a large increase in telomerase activity when analyzed separately (i.e., apart from whole tissue samples), suggesting that they are the cell type mainly responsible for the increases evident in telomerase activity/protein, and hence telomere length, following axotomy.
Figure 4-9. FACS-isolation of microglia from axotomized and control facial nuclei. Facial nuclei (axotomized or control) were pooled from rats (5 days post-axotomy) and subjected to FACS analysis. Antibodies to CD11b/c and CD45 were used to isolate microglia. A and B are the negative control (no antibodies used); C and D are the CD11b/c antibody only; E and F are the CD45 antibody only; G and H are the axotomized facial nuclei; I and J are the control facial nuclei. Cells within R1 were selected and sorted via R2 gating to isolate microglia.
Figure 4-10. TRAP analysis image used for measurement of telomerase activity in FACS-isolated facial nuclei.

Figure 4-11. Densitometric quantitation of telomerase activity in FACS-isolated facial nuclei. Total protein was isolated from FACS-isolated microglia from axotomized (A) and control (C) facial nuclei (day 5 post-axotomy, six independent animals, two separate experiments). IC = internal control. Telomerase activity (arbitrary units) within FACS-isolated microglia from axotomized facial nuclei on day 5 post-axotomy was increased by 254.71% compared to the control facial nuclei.
Discussion

We have shown that facial nerve axotomy results in a probable increase in telomere length and telomerase protein, and a definite increase in telomerase activity in the axotomized facial nucleus. A definite increase in telomerase activity also occurs in FACS-isolated activated microglia from the axotomized facial nucleus. These results support the hypothesis that maintenance and extension of telomere length occurs in activated microglia that accumulate in axotomized rat facial nuclei. Telomere extension is likely the result of the observed increases in telomerase protein and activity. Our findings suggest that dividing microglial cells of the CNS compensate for replication-induced telomere shortening by up-regulating telomerase.

A total of 10 facial nuclei were pooled for use in Southern blot analysis. This pooling of tissue samples was necessary due to their small size (approximately 7 mg wet weight each) and in order to load sufficient quantities of DNA in order to generate a signal during the Southern blot detection process. Axotomy caused a trend towards an increase in telomere length (in the longest, mean, and shortest) in the axotomized facial nuclei, compared to the control facial nuclei. A concomitant increase in both telomerase activity and telomerase protein was also observed in axotomized facial nuclei. Since microglia are the only cells known to divide in response to axotomy (Graeber et al., 1988), these findings suggest that microglia are utilizing telomerase to regulate telomere length in vivo during periods of high proliferation. We believe that this presumed increase in microglial telomere length in the axotomized facial nuclei compensates for the shortening of telomeres that would otherwise occur in the absence of such telomerase activity. Since FACS-isolated microglia from the axotomized facial nuclei exhibit an increase in telomerase activity, the increase in telomere length evident within whole
facial nuclei may be attributable to the increased telomerase activity present within microglia. It remains possible that other cell types may be present which up-regulate expression, and hence activity, of telomerase following axotomy. Telomerase activity increased the most from day 1 to 4, and peaked on day 7 post-axotomy, representing a maximal increase in activity of 104.05%. In view of proliferation data from previous studies (Graeber et al., 1988; Kreutzberg, 1966; Streit and Kreutzberg, 1988; Svensson et al., 1994), which showed a peak in microglial proliferation on day 3 post-axotomy, our findings suggest that the increase in telomerase activity evident within the first 4 days following axotomy is to prepare microglia for their concomitant proliferative burst (occurring on days 2 to 4 post-axotomy). Telomerase activity continued to rise from day 4 to 7 as well, indicating the presence of enzymatic activity to lengthen telomeres, if needed. From 7 to 10 days post-axotomy, telomerase activity declined, possibly since additional proliferation does not occur during this time period (Graeber et al., 1988; Kreutzberg, 1996; Streit and Kreutzberg, 1988; Svensson et al., 1994), and thus telomere length would likely remain stable, and hence telomerase activity would not be required. Analysis of telomerase activity in naïve (i.e., non-axotomized) animals showed no difference in telomerase activity between the two sides, as expected. The processivity of the telomerase enzyme (Greider, 1991) (i.e., the quantity of telomeric repeats processed by the enzyme, as determined by banding pattern on gel), in both whole facial nuclei and in microglia FACS-isolated from facial nuclei, correlated well with measured telomerase activity. Lanes possessing an intense initial band (immediately above the internal control band) always contained a large quantity/intensity of small telomerase products, as well as a small quantity/intensity of larger products, and are indicative of samples with low
enzyme processivity (i.e., control side of injured facial nucleus). The presence of the intense initial band indicates that, most of the time, telomerase added only a single hexanucleotide telomeric repeat onto the end of the TS primer before dissociating. Thus, most of the measured telomerase activity was contributed by the presence of the first few initial bands. In these lanes, telomerase apparently is only able to generate small products and lacks the ability to create larger products, which are clearly present in lanes with high telomerase processivity (i.e., axotomized side of injured facial nucleus).

There was a two-fold increase in the number of microglia isolated by FACS from the axotomized facial nuclei (i.e., 1,334) compared to the control facial nuclei (i.e., 669), which was expected since microglia are known to proliferate in response to axotomy (Graeber et al., 1988; Kreutzberg, 1996). Since TRAP analysis is a PCR-based assay, it enabled the measurement of telomerase activity from such small numbers of cells. FACS-isolated microglia from facial nuclei exhibited an increase in telomerase activity (by 255%) compared to the control facial nuclei, and thus the increase in telomerase activity evident within whole facial nuclei tissue samples is most likely attributable to microglia. Importantly, telomerase activity in FACS-isolated microglia (255% higher in axotomized side on day 5 post-axotomy) is 270% higher compared to that in whole facial nuclei tissue samples (69% higher in axotomized side on day 4 post-axotomy). Thus, when telomerase activity is measured in whole tissue samples, cells other than microglia which may have low telomerase activity, likely account for the overall decrease in enzymatic activity.

Few studies have examined telomere length or telomerase activity in the brain, and most published research in this area has focused on CNS tumors and neural precursor
cells. We have previously shown that telomere shortening occurs in cultured rat microglia in vitro (Flanary and Streit, 2004), and in rat cerebellum and cortex in vivo in the presence of low levels of telomerase activity (Flanary and Streit, 2003). One study reported that telomerase activity is high in mouse cortex during embryonic development, but sharply decreases during postnatal development up to three months of age (Klapper et al., 2001). Telomerase activity is also high in rat oligodendrocyte precursor cells, but declines during their differentiation into mature oligodendrocytes (Caporaso and Chao, 2001). Human neural precursor cells express low levels of telomerase at early passages, with levels declining to undetectable levels at later passages (greater than 20 population doublings). In contrast, rodent neural precursor cells express high levels of telomerase at both early and late passages (Ostenfeld et al., 2000). Telomerase has been found to be expressed in all brain regions shortly after birth, but becomes restricted to neural stem cells within the subventricular zone and olfactory bulb in the adult mouse brain (Caporaso et al., 2003). Likewise, another study reported that telomerase activity is high in rodent neurons during embryonic and early postnatal development, but decreases subsequently (Fu et al., 2002). The latter study also found that suppression of telomerase expression in cultured embryonic hippocampal neurons increased their vulnerability to apoptosis and excitotoxicity, suggesting that telomerase may play roles other than telomere maintenance. Induction of telomerase in neurons has been found to exhibit neuroprotective properties in experimental animal models of neurodegenerative disorders (Mattson, 2000). Another study reported the induction of telomerase mRNA in cortical neurons following ischemia (Kang et al., 2004). Thus, telomerase appears to play critical roles during embryonic development and following brain injury, and it may be
neuroprotective in non-dividing neurons by performing functions unrelated to telomere length maintenance, such as repair of telomeres damaged by free radicals (von Zglinicki, 2002). The results from our current study in rats also suggest a link between telomerase and neuroprotection, in that increased telomerase activity may prevent microglial senescence thereby ensuring sustained glial support of injured neurons. An acute increase in activated microglia in vivo following axotomy not only serves a beneficial role, but is also a crucial component of the regenerative process, since microglia divide in response to the injury, surround and ensheath injured motor neurons, and provide them with trophic support (Streit, 2002b). However, if multiple bouts of proliferation occurs (e.g., in response to repeated injury), this may result in an accelerated rate of telomere shortening in microglia, which may hasten their entry into replicative senescence, and may thereby limit both the quantity and quality of glial support they are able to provide to neurons. Recently, astrocytes and microglia have been shown to express telomerase immunoreactivity in vivo following ischemic or kainite-induced brain injury (Baek et al., 2004; Fu et al., 2002). We also performed immunohistochemistry for the detection of telomerase reverse transcriptase, but these experiments were unsuccessful in that no specific immunoreactivity was observed. The experiments were performed with two different anti-telomerase antibodies (rabbit anti-telomerase antibody, NB 100-141, from Novus Biologicals, Littleton, CO; or rabbit anti-telomerase antibody, EST-21A, from Alpha Diagnostics, San Antonio, TX), using both 4% paraformaldehyde and 10% formalin fixation with and without antigen retrieval in 0.01 M citrate buffer.

We have shown by Western blot analysis that telomerase protein is increased in the axotomized facial nucleus compared to the control side. On day 10, there is over a 100%
increase in telomerase activity, which corresponds to, and is likely caused by, the higher rate of decline of telomerase protein in the control compared to the axotomized side from day 7 to 10 post-axotomy. It is unknown what causes telomerase protein to decrease at a slightly faster rate in the control side (relative to the axotomized side) from day 7 to 10 post-axotomy. On day 10, the quantity of telomerase protein is markedly higher than day 1 in the axotomized side, but is slightly lower in controls relative to day 1, suggesting that telomerase protein (and activity) are necessary in the axotomized side up to at least day 10 post-axotomy. The largest increase detected was on day 4 post-axotomy (45.13% increase) (p<0.03), followed by day 7 (37.01% increase). This data corresponds well with the microglial proliferative burst after axotomy in that the largest increase in the production of telomerase protein is coincident with the maximal number of microglial cells present 4 days after the axotomy. The increase in telomerase protein also shows good temporal correspondence with a large increase in telomerase activity suggesting that increased enzymatic activity may be due to the observed increase in telomerase protein. Specifically, the increase in telomerase protein preceded the increase in telomerase activity, since the percent increase in protein peaked on day 4 (45.13% higher in axotomized facial nuclei), whereas activity peaked on day 7 (104.05% higher in axotomized facial nuclei). This data suggests that telomerase is translationally-regulated, at the least. Interestingly, the temporal profiles of telomerase protein followed parallel patterns in the axotomized and control facial nuclei. Both axotomized and unoperated control facial nuclei showed increases in telomerase protein from day 1 to 4, and a decrease from day 4 to 10. This suggests that unilateral axotomy can produce
contralateral effects, an interesting phenomenon that has been noted before but remains exceedingly difficult to define because of its subtlety and inconsistency.

The findings from our previous studies in vitro (Flanary and Streit, 2004) provided an impetus to further characterize telomere length and telomerase activity in the facial nucleus following repeated axotomies to induce continuous mitogenic stimulation. We found that microglia exposed to continuous mitogenic stimulation with GM-CSF in vitro show a dramatic increase in telomere length during the first few days post-stimulation, followed by rapid telomere shortening and senescence. In the current study, a similar situation is observed in that after microglia undergo a short burst in proliferation following axotomy, a resultant increase is observed in telomere length. Telomere shortening may become evident when microglia are subjected to multiple rounds of proliferative bursts induced by repeated axotomies. These experiments will be the focus of future studies, and may support the hypothesis that repeated brain injury could lead to microglial senescence.
CHAPTER 5
ALPHA-TOCOPHEROL (VITAMIN E) INDUCES RAPID, NON-SUSTAINED PROLIFERATION IN CULTURED RAT MICROGLIA

Introduction

Microglial Activation

Activation of microglial cells is a critical component of the brain’s response to injury. It is particularly prominent after acute lesions when it occurs rapidly and is characterized, among other things, by a dramatic increase in microglial cell numbers (Graeber et al., 1988; Kreutzberg, 1996). Granulocyte macrophage-colony stimulating factor (GM-CSF) has long been known to be an effective microglial mitogen in vitro (Giulian and Ingeman, 1988; Suzumura et al., 1990), and it has also been implicated as a stimulator of microglial mitosis after acute injuries (Raivich et al., 1991, 1994). From a functional point of view, it can be reasonably surmised that the rapid proliferation of microglial cells shortly after a CNS lesion occurs because greater numbers of these cells are required to initiate the complex processes of wound healing and tissue repair (Streit et al., 2000).

Microglial activation is also thought to occur with normal aging and in age-related neurodegenerative diseases, such as Alzheimer’s disease (AD) (McGeer et al., 1987; Rogers et al., 1988; Streit and Sparks, 1997; Akiyama et al., 2000; McGeer and McGeer, 2001). However, unlike the microgliosis observed after acute CNS injuries, there is no evidence to show that age- or AD-related microglial activation is accompanied by increased cell division. Quite to the contrary, there is evidence showing that increased
microglial cell death, as well as microglial structural abnormalities (microglial dystrophy) are prominent features of the aged and AD brain (Lassmann et al., 1995; Yang et al., 1998; Jellinger and Stadelmann, 2000; Streit et al., 2004a). The latter observations have raised the possibility that a loss of microglial cells or of microglial cell function could contribute to the development of age-related neurodegenerative diseases (Streit, 2002a, b). Notwithstanding these relatively recent findings, the notion that chronic microglial activation (often referred to as neuroinflammation) is detrimental and a contributing factor in AD pathogenesis has become widely accepted and has resulted in the use of anti-inflammatory regimens as potential treatments (Akiyama et al., 2000). The neuroinflammation concept has fueled the idea that oxidative stress increases in the aging brain, in part because activated microglia in vitro produce reactive oxygen species (Colton and Gilbert, 1987), and it is therefore not surprising that antioxidants have been, and continue to be, explored as potential anti-aging treatments (Jackson et al., 1988; Sano et al., 1997; O'Donnell and Lynch, 1998; Milgram et al., 2002; Devi and Kiran, 2004).

**Function of Vitamin E**

Vitamin E is the most effective lipid-soluble antioxidant in biological membranes, and it acts to stabilize lipid membranes and prevent propagation of free radical damage (Halliwell and Gutteridge, 1985). Vitamin E (i.e., alpha-tocopherol) has an organic structure possessing two aromatic rings and a hydrocarbon tail. Cell surface receptors exist for the binding and uptake of vitamin E (Kaempf-Rotzoll et al., 2003; Meier et al., 2003), however, very few studies have examined which specific receptors are responsible for its uptake. Specific receptor sites have been found for vitamin E on bovine adrenocortical cells (Kitabchi et al., 1980) human erythrocytes (Kitabchi and Wimalasena, 1982), and rat hepatocytes (Murphy and Mavis, 1981). Dietary
supplementation of vitamin E has shown benefits for immune cell function (Bendich, 1988; Tengerdy, 1989), as well as cognitive performance and neuroprotection (Socci et al., 1995; Perrig et al., 1997; Behl and Holsboer, 1998; Joseph et al., 1998; Joseph et al., 1999; Martin et al., 1999; Behl and Moosmann, 2002; Grundman and Delaney, 2002; Martin et al., 2002). There are completed and ongoing human clinical trials using vitamin E as a potential treatment for AD (Sano et al., 1997). The effects of vitamin E on cultured microglial cells have been studied, and most of this work, consistent with the idea that microglial activation is a harmful process, has been interpreted to show that vitamin E suppresses microglial activation (Heppner et al., 1998; Li et al., 2001; Egger et al., 2001, 2003; Gonzalez-Perez et al., 2002; Godbout et al., 2004; Grammas et al., 2004). Thus, there appears to be a consensus currently that vitamin E may provide some neuroprotection by deactivating microglial cells. In the current study, we have investigated long term effects of vitamin E on primary rat microglial cell cultures with the goal of determining its effects on cellular aging and proliferation kinetics. The results reveal that vitamin E is a most potent microglial mitogen that stimulates dramatic microglial proliferation \textit{in vitro}. Not unexpectedly, we have also found concomitant shortening of telomere length and loss of telomerase activity in these cultures.

**Materials and Methods**

**Culturing of Microglia**

Microglia were isolated as described in Chapter 1.

**Treatment of Microglial Cells**

Microglia were treated on day 0 with either 10.2 nM (0.15 $\mu$g/mL) recombinant rat granulocyte-macrophage colony stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN), DL-$\alpha$-tocopherol acetate (Sigma-Aldrich, St. Louis, MO; catalog #
T3376-25G) dissolved in 100% ethanol (EtOH) and sterile-filtered at concentrations of 20.0 μM (9.5 μg/μL), 105.7 μM (50 μg/μL), or 500.0 μM (236 μg/μL), DL-α-lipoic acid (Sigma-Aldrich, St. Louis, MO; catalog # T-1395) dissolved in 100% EtOH and sterile-filtered at concentrations of 20.0 μM (9.5 μg/μL), 105.7 μM (50 μg/μL), or 500.0 μM (236 μg/μL), α-tocopherol and α-lipoic acid (same concentrations as above), or an identical volume of sterile-filtered 100% EtOH as a vehicle control (i.e., 10 μL/1 mL media). In all experiments, media (and respective treatment) was changed as needed (usually every 3 to 4 days).

**Determination of Cell Proliferation**

Cell proliferation was measured as described in Chapter 1.

**Determination of Interleukin-1β Production**

To determine production of interleukin-1 beta (IL-1β) by cultured microglia, the Quantikine M rat IL-1β immunoassay was used (R & D Systems, Minneapolis, MN). Quantitative determination of rat IL-1β concentrations in cell culture supernatants was performed according to the manufacturer’s recommended protocol.

**Determination of Telomere Length**

Telomere length was measured as described in Chapter 1.

**Determination of Telomerase Activity**

Telomerase activity was measured as described in Chapter 1.

**Statistical Analysis of Data**

Statistical analyses were performed as described in Chapter 3.
Results

Microscopic Examination of Cultured Rat Microglia at Various Times and Treatments

Representative photographs of cultured rat microglia on the indicated day under various treatment conditions are shown in Fig. 5-1. Microglia treated with 1% EtOH (vehicle controls) gradually decline in cell number from day 2 to 7 and were stable from day 7 to 14. The most dramatic effect is evident in microglia treated with 105.7 μM α-tocopherol (vitamin E). These cells underwent rapid and sustained proliferation from day 0 to 2, compared to EtOH controls, peaked in cell number at around day 7, and then declined substantially in cell number from day 7 to 14. Cells treated with 500 μM α-lipoic acid (LA) exhibited a gradual decline in cell number from day 2 to 14.

Vitamin E Induces Cell Proliferation in Cultured Rat Microglia

Cell proliferation (as determined by MTT) of cultured rat microglia from day 2 to 27 under various treatment conditions is shown in Fig. 5-2. As reported before (Flanary and Streit, 2004), treatment of microglia with 10.2 nM recombinant rat granulocyte-macrophage colony stimulating factor (GM-CSF) resulted in a significant and sustained increase in cell proliferation compared to other treatments from day 2 to 27. Proliferation was significantly higher in GM-CSF-treated microglia (compared to all other groups, except vitamin E) on day 7 [p<0.001 (compared to EtOH vehicle control and LA-treated cells)], day 14 [p<0.05 (compared to vitamin E and E & LA-treated cells); p<0.001 (compared to EtOH vehicle control and LA-treated cells)], and day 27 [p<0.01 (compared to EtOH vehicle control-treated cells); p<0.001 (compared to vitamin E, LA, and E & LA-treated cells)]. Treatment of microglia with 105.7 μM α-tocopherol (vitamin E)
Figure 5-1. Representative micrographs of cultured rat microglia under various treatment conditions: [100% EtOH (10 μL/1 mL media) as vehicle control, 105.7 μM α-tocopherol acetate (i.e., vitamin E: Vit E), 500.0 μM α-lipoic acid (LA), or both Vit E and LA (same concentrations as above)]. A, B, C = 100% EtOH-treated cells on days 2, 7, and 14; D, E, F = Vit E-treated cells on days 2, 7, and 14; G, H, I = LA-treated cells on days 2, 7, and 14; J, K, L = E & LA-treated cells on days 2, 7, and 14. All photographs are at 10X magnification. Scale bar = 20 μm.

produced the greatest significant increase in microglial proliferation from day 2 to 7 (p<0.001), compared to all other treatment groups. However, on day 14, proliferation dropped to levels below that of GM-CSF-treated microglia, only to peak again significantly on day 21 [p<0.01 (compared to E & LA-treated cells); p<0.001 (compared
to EtOH vehicle control and LA-treated cells), and subsequently decline on day 27. These results correlate well with the photographs presented in Fig. 5-1 in that microglia were shown to increase in cell number from day 2 to 7 and decrease substantially in cell number from day 7 to 14. The combination treatment of vitamin E and α-lipoic acid (E & LA) resulted in an intermediate cell proliferation between that of α-lipoic acid (LA) or vitamin E alone. Microglia treated with LA exhibited cell proliferation below that of EtOH controls on all days examined. On day 2, microglial cells treated with E & LA had a significant higher cell proliferation than LA alone (p<0.05), while on day 14 exhibited a higher proliferation than both EtOH controls (p<0.001) and LA (p<0.001).

Figure 5-2. Cell proliferation (as determined by MTT assay) of cultured rat microglia on the indicated days under various treatment conditions: [100% EtOH (10 μL/1 mL media) as vehicle control, 105.7 μM α-tocopherol acetate (i.e., vitamin E: Vit E), 500.0 μM α-lipoic acid (LA), both Vit E and LA (E & LA) (same concentrations as above), or 10.2 nM recombinant rat granulocyte-macrophage colony stimulating factor (GM-CSF)]. Treatment with 105.7 μM vitamin E produced the greatest increase in microglial proliferation.
The proliferation rate (as determined by BrdU incorporation) of cultured rat microglia from day 1 to 12 under various treatment conditions is shown in Fig. 5-3. Treatment of microglia with 10.2 nM recombinant rat granulocyte-macrophage colony stimulating factor (GM-CSF) resulted in a sustained significant increase in proliferation rate from day 2 to 12 compared to other treatments (except vitamin E-treated microglia from day 2 to 12). Treatment of microglia with 105.7 μM α-tocopherol (vitamin E) produced the greatest increase in proliferation rate from day 2 to 12. Proliferation rate was significantly higher in vitamin E-treated microglia (compared to all other groups) on day 5 [p<0.01 (compared to EtOH vehicle control and GM-CSF-treated cells); (p<0.001 compared to LA and E & LA-treated cells)], day 7 (p<0.001 compared to all other treatments), and day 12 (p<0.001 compared to all other treatments). These results correlate well with the data presented in Fig. 5-1 and 5-2. The combination treatment of E & LA resulted in an intermediate proliferation rate between that of LA or vitamin E alone. Microglia treated with LA exhibited proliferation rates below that of EtOH controls on all days examined, as was seen in the MTT assay.

Quantitation of cell proliferation of cultured rat microglia at 48 hours under various treatment conditions is shown in Fig. 5-4. Treatment of microglia with 20 μM vitamin E resulted in the highest increase in proliferation compared to all other treatment groups and concentrations. Notwithstanding 20 μM E & LA, treatment of microglia with 20, 105.7, and 500 μM vitamin E yielded the top 3 highest increases in cell proliferation, respectively, compared to other treatment groups. Proliferation was significantly higher (p<0.01 to 0.001) in 20 μM vitamin E-treated microglia, compared to all other groups (except 20 μM E & LA-treated cells). Treatment of microglia with 20 μM E and LA
Figure 5-3. Proliferation rate (as determined by BrdU incorporation over 2 hours) of cultured rat microglia on the indicated days under various treatment conditions: [100% EtOH (10 μL/1 mL media) as vehicle control, 105.7 μM α-tocopherol acetate (i.e., vitamin E: Vit E), 500.0 μM α-lipoic acid (LA), both Vit E and LA (E & LA) (same concentrations as above), or 10.2 nM recombinant rat granulocyte-macrophage colony stimulating factor (GM-CSF)]. Treatment with 105.7 μM vitamin E produced the greatest increase in microglial proliferation, and was significantly higher than EtOH, LA, E&LA, and GM-CSF on days 5, 7, and 12 (p<0.001).

resulted in the second-highest increase in proliferation in all instances, and was significantly higher (p<0.01 to 0.001) than all other treatment groups (except 20 μM and 105.7 μM vitamin E-treated cells). The combination treatment of E & LA resulted in an intermediate proliferation between that of LA or vitamin E alone. In all treatment groups, there was a strong inverse correlation between proliferation and treatment concentration, such that cell proliferation was highest when treated with the lowest concentration (i.e., 20 μM) of each treatment, and vice versa.
Telomere Length Analysis in Vitamin E-Treated Cultured Rat Microglia

Quantitation of telomere length (via Southern blot analysis) in microglia under various treatment conditions from day 0 to 7 is shown in Fig. 5-5. In EtOH controls, both the longest and shortest telomeres underwent little change from day 0 to 7, while the mean telomeres shortened from day 0 to 2, and subsequently lengthened from day 2 to 7. In both vitamin E-treated and α-lipoic (LA) acid-treated microglia, the longest telomeres also underwent little change from day 0 to 7, with the shortest telomeres gradually decreasing in length, while the mean telomeres underwent a moderate/large (respectively) decrease in length from day 0 to 7. On day 7 in microglia treated with LA, the mean telomeres were over 100% shorter compared to EtOH controls on the same day. In microglia treated with a combination of vitamin E and LA, the shortest telomeres
remained relatively unchanged, however, both the largest and mean telomeres exhibited large decreases in length from day 2 to 7 and day 0 to 7, respectively. On day 7 in microglia treated with vitamin E and LA, both the longest and mean telomeres were well over 100% shorter compared to EtOH controls on the same day. Overall, telomere lengths were shorter on both day 2 and 7 in microglia in all treatment groups compared to EtOH controls on corresponding days.

![Figure 5-5](image.png)

Figure 5-5. Densitometric quantitation of telomere length in cultured rat microglia on the indicated days under various treatment conditions: [100% EtOH (10 μL/1 mL media) as vehicle control, 105.7 μM α-tocopherol acetate (i.e., vitamin E: Vit E), 500.0 μM α-lipoic acid (LA), or both Vit E and LA (same concentrations as above)].

**Telomerase Activity Analysis in Vitamin E-Treated Cultured Rat Microglia**

A representative photograph of a TRAP analysis image used for determination of telomerase activity in cultured rat microglia is shown in Fig. 5-6. Quantitation of telomerase activity in microglia under various treatment conditions from day 0 to 14 is shown in Fig. 5-7. In EtOH controls, telomerase activity significantly increased
(p<0.001) from day 0 to 7, then significantly declined (p<0.001) from day 7 to 14. Treatment with $\alpha$-lipoic acid (LA) also caused an initial significant increase (p<0.001) in telomerase activity from day 0 to 7, with a subsequent significant decrease (p<0.001) from day 7 to 14. The pattern of telomerase activity in LA-treated cells mirrored that in EtOH controls, and LA-treated cells exhibited lower levels of activity at all times points relative to controls. In vitamin E-treated microglia, telomerase activity significantly increased (p<0.001) from day 0 to 2, declined from day 2 to 7, then increased again from day 7 to 14. In microglia treated with a combination of vitamin E and LA, telomerase activity significantly declined (p<0.001) from day 0 to 14. In relation to EtOH controls, every treatment suppressed telomerase activity except for vitamin E on day 14. The only treatment that yielded an overall increase in telomerase activity from day 0 to 14, and was the treatment that resulted in the highest telomerase activity at day 14, was vitamin E.

Quantitation of telomerase activity in cultured rat microglia at 48 hours under various treatment conditions is shown in Fig. 5-8. Telomerase activity was high in vitamin E-treated microglia at 20 and 105.7 $\mu$M concentrations, but significantly decreased (p<0.001) by over two-fold when treated with 500 $\mu$M vitamin E. Telomerase activity was also high in microglia treated with 105.7 $\mu$M $\alpha$-lipoic acid (LA), but was approximately 33% and 50% lower (p<0.001) in cells treated with 20 $\mu$M and 500uM LA, respectively. In microglia treated with a combination of vitamin E and LA, telomerase activity was moderately high at 20 $\mu$M concentrations, but significantly decreased (p<0.001) by approximately two-fold when treated with 105.7 or 500 $\mu$M LA. In all instances, telomerase activity decreased with increasing treatment concentration, except in LA-treated microglia from 20 to 105.7 $\mu$M.
Figure 5-6. Representative TRAP analysis image used for measurement of telomerase activity in cultured rat microglia. Shown here is the TRAP image of microglia at 48 hours under various treatment conditions: [100% EtOH (10 μL/1 mL media) as vehicle control, 20.0, 105.7, or 500.0 μM α-tocopherol acetate (i.e., vitamin E: Vit E), 20.0, 105.7, or 500.0 μM α-lipoic acid (LA), or both Vit E and LA (same concentrations as above)]. Neg = telomerase-negative control; Pos = telomerase-positive control; IC = internal control.
Figure 5-7. Quantitation of telomerase activity in cultured microglia on the indicated
days under various treatment conditions: [100% EtOH (vehicle control),
105.7 μM α-tocopherol acetate (i.e., vitamin E: Vit E), 500.0 μM α-lipoic
acid (LA), or both Vit E and LA (same concentrations as above)].

**Interleukin-1 Beta Production in Cultured Rat Microglia**

Quantitation of IL-1β production by cultured microglia under various treatment
conditions from day 2 to 7 is shown in Fig. 5-9. Treatment of microglia with either EtOH
(control), vitamin E, α-lipoic acid (LA), or E & LA caused a significant increase
(p<0.001) in production of IL-1β from day 2 to 7. In microglia treated with a
combination of vitamin E and LA, production of IL-1β was significantly higher
(p<0.001) compared to all other treatments at each time point measured. In addition,
each treatment group was significantly higher (p<0.001) than EtOH controls on each day,
except for vitamin E alone and LA alone on day 7. Importantly, IL-1β production in
vitamin E-treated microglia was significantly lower than all other groups on day 7.
Figure 5-8. Quantitation of telomerase activity in cultured microglia at 48 hours under various treatment conditions: [100% EtOH (vehicle control), 20.0, 105.7, or 500.0 μM α-tocopherol acetate (i.e., vitamin E: Vit E), 20.0, 105.7, or 500.0 μM α-lipoic acid (LA), or both Vit E and LA (same concentrations as above)].

Figure 5-9: Quantitation of interleukin-1β production by cultured rat microglia on the indicated days under various treatment conditions: [100% EtOH (10 μL/1 mL media) as vehicle control, 105.7 μM α-tocopherol acetate (i.e., vitamin E: Vit E), 500.0 μM α-lipoic acid (LA), or both Vit E and LA (same concentrations as above)]. A rat IL-1β positive control (included in the kit) yielded 174.5 pg/mL.
Discussion

This study is the first to report the mitogenic effects of α-tocopherol on microglia, and it shows that treatment of cultured rat microglia with vitamin E for 7 days resulted in a very significant increase in cell numbers. The magnitude of the mitogenic effect of vitamin E on microglia is approximately twice that of the known microglial mitogen, GM-CSF, and thus, vitamin E may be considered to be at present the most potent microglial mitogen known. As expected, and in line with our prior work (Flanary and Streit, 2004), the increase in microglial proliferation was accompanied by a decrease in telomere length in the presence of decreasing telomerase activity. In addition, our results show that the mitogenic effect of vitamin E on microglial proliferation is independent of its anti-oxidative action since cultures treated in parallel with the antioxidant α-lipoic acid did not show a proliferative response.

Microglia treated with vitamin E underwent a massive increase in cell number from day 2 to 7 and then declined from day 7 to 14, correlating well with BrdU incorporation over the same time period. These findings underscore the transient nature of this effect in vitro and beg the question whether vitamin E can sustain microglial mitosis in vivo in the long term. In vitro, vitamin E-induced microglial proliferation falls to levels below those of controls on day 27, supporting the notion that these cells have exhausted their replicative capacity at this point, and are likely nearing replicative senescence. A similar situation existed for microglia treated with vitamin E & LA in that they, too, exhibited proliferation that was significantly higher than in EtOH controls, however, following day 7 this proliferation declined steadily and eventually fell below levels seen in controls on day 27. In LA-treated microglia, cell proliferation was lower than in controls at all time
points examined. In addition, cell proliferation in E & LA-treated microglia was lower compared to vitamin E-treated cells on all days examined, and was significantly lower (p<0.001) than vitamin E-treated cells on day 2, 7, and 21. Collectively, these observations suggest that LA suppressed microglial proliferation. On the other hand, GM-CSF-treated microglia also exhibited an early significant increase in cell proliferation compared to vehicle controls, yet these cells were able to sustain their proliferative activity through day 27. This suggests that GM-CSF-treated microglia may be able to proliferate longer before entering replicative senescence compared to vitamin E-treated microglia, perhaps because GM-CSF-treated microglia divided at a lower level relative to vitamin E-treated cells. Vitamin E-treated microglia seemingly “used up” most of their proliferative potential during the first week of in vitro growth.

In our previous studies (Flanary and Streit, 2004), we found that GM-CSF-treated cultured rat microglia exhibited higher cell proliferation, greater telomerase activity, and increased telomere length compared to controls during the first 2 days of in vitro growth, presumably in an effort to compensate for the loss of telomeric repeats that would otherwise occur during the period of high proliferation. Vitamin E-treated microglia exhibited an even greater increase in proliferation from day 2 to 7 than GM-CSF-treated cells, and also showed an increase in telomerase activity. However, telomere length in vitamin E-treated microglia decreased from day 0 to 7, suggesting that the very high rate of proliferation caused by vitamin E exposure resulted in the inexorable shortening of telomeres despite an increase in telomerase activity during the same time period.

The processivity of telomerase (Greider, 1991), as determined by banding pattern on gel, correlated well with overall telomerase activity. Lanes possessing an intense
initial band (immediately above the internal control band), which always contained a large quantity of small telomerase products and a small quantity of larger products, represent samples with low enzyme processivity (e.g., 500 μM vitamin E). The presence of an intense initial band indicated that most of the time telomerase added only a single hexanucleotide telomeric repeat onto the end of the TS primer before disassociating. Thus, a great proportion of the overall measured telomerase activity was contributed by the presence of the first few initial bands. In these lanes, telomerase could only generate small products and lacked the ability to create larger ones (which are clearly present in lanes with high telomerase processivity). The presence of low enzyme processivity usually coincided with lower overall telomerase activity in our samples.

Production of interleukin-1β, one type of cytokine that mediates the inflammatory response, was significantly decreased in vitamin E-treated microglia in vitro. This suggests that vitamin E significantly suppressed microglial activation in vitro, in terms of IL-1β production (on day 7), and is consistent with previous research showing that vitamin E may provide neuroprotection by suppressing intracellular signaling events necessary to induce microglial activation (Heppner et al., 1998; Li et al., 2001; Egger et al., 2001, 2003; Gonzalez-Perez et al., 2002; Godbout et al., 2004; Grammas et al., 2004), and that vitamin E decreases/suppresses IL-1B production (Akeson et al., 1991; Devaraj et al., 1996; Devaraj and Jialal, 1999; Pathania et al., 1999; Gonzalez et al., 2001). Interleukin-1β production was significantly higher in E & LA-treated microglia compared to all other treatments at each timepoint measured, despite the significantly lower IL-1β levels produced with either vitamin E or LA treatments alone. This suggests that a synergistic effect occurred (in terms of increased IL-1β production) when both
antioxidants were used in combination (Scholich et al., 1989; Haramaki et al., 1993; Stoyanovsky et al., 1995), that a combinational treatment is more efficient at activating microglia in vitro, and that LA decreased/eliminated the ability of vitamin E alone to significantly lower IL-1β levels. In all instances, IL-1β production increased from day 2 to 7, suggesting that IL-1β levels increase as cells age in vitro and progress toward senescence. This correlates with previous research indicating that activated interleukin-1 positive human microglia increase with age (Sheng et al., 1998).

A wealth of previously-published research supports our current findings (i.e., that vitamin E exerts mitogenic effects). One of the earliest reports showed that vitamin E enhanced guinea pig smooth muscle cell proliferation (Miller et al., 1980). Previous research has also indicated that vitamin E acts as a potent mitogen for peripheral macrophages (Oonishi et al., 1995) and lymphocytes (Meydani et al., 1986; Roy et al., 1991; Sakai and Moriguchi, 1997). Clearly, vitamin E plays an important role as a mitogenic agent in a variety of cell types. Surprisingly, none of the studies published thus far have reported the increase in microglial proliferation that we observed. Heppner et al. (1998) noted that “cell numbers of microglia treated with vitamin E remained stable within 7 days in vitro” and that “vitamin E was shown to protect microglial cell cultures from excessive loss of viable cells;” however, this study only used 10 μg/mL vitamin E treatment, which may account for the absence of microglial proliferation. Heppner et al. (1998) concluded that since vitamin E treatment of primary rat microglia cells caused them to down-regulate expression of adhesion molecules associated with microglial activation, that scavenging of free radicals (via vitamin E) may prevent/reverse microglial activation. Similarly, a study by Li et al. (2001) found that vitamin E treatment inhibited
LPS-induced activation in the N9 murine microglial cell line as determined by down-regulation of inflammatory cytokines (i.e., IL-1α, TNF-α), and nitric oxide via a p38 mitogen-activated protein kinase and nuclear factor kappa B-dependent pathway; however there is no mention of changes in microglial cell numbers following vitamin E treatment. Studies by Egger et al. (2001, 2003) found that treatment of both primary porcine microglia and the BV-2 murine microglial cell line with vitamin E (up to 100 μM) attenuated superoxide production and transcription of cyclo-oxygenase 2; however, there is no mention in either report of changes in microglial cell numbers following vitamin E treatment. Other studies have found that vitamin E treatment reduced LPS-induced activation in primary murine microglial cells as determined by decreased production of the inflammatory cytokine IL-6 (Godbout et al., 2004) and prostaglandin E(2) production (Grammas et al., 2004), but make no reference to potential mitogenic effects.

In the current study, the mitogenic effect of vitamin E on microglia is most dramatic in that it exceeds the effect of the well-known microglial mitogen, GM-CSF (Giulian and Ingeman, 1988) and thus, vitamin E may be considered to be (at present) the most powerful known mitogen for microglia in vitro. We have been able to consistently and repeatedly reproduce this effect using different batches of both microglial cultures, and alpha tocopherol lots/stock solutions, in our laboratory. Even though the CNS is generally considered a post-mitotic tissue, it is important to note that microglia do retain a robust proliferative potential, especially under conditions of CNS injury, as shown by studies using ³H-thymidine incorporation (Graeber et al., 1988; Kreutzberg, 1996). This increase in microglial cell numbers following neuronal injury likely occurs because
additional cells are needed to assist injured neurons (Streit, 2002b). Vitamin E may therefore act as a neuroprotective agent by not only suppressing microglial activation (in terms of cytokine production), but also by increasing microglial cell numbers. Although an increase in cell numbers is one aspect of microglial activation, this feature by itself may not be a harmful or detrimental process to microglia themselves or to bystander cells. If vitamin E does result in an increase in microglial proliferation in vivo, then this may provide an additional mechanism (in addition to scavenging free radicals) to explain why vitamin E supplementation has been shown to be beneficial in rodent models of stroke (Stohrer et al., 1998; van der Worp et al., 1998; Tagami et al., 1999; Noguchi et al., 2001; Gonzalez-Perez et al., 2002; Chaudhary et al., 2003; Ikeda et al., 2003; Niu et al., 2003) and brain injury (Yoshida et al., 1985; Clifton et al., 1989; Inci et al., 1998; Ikeda et al., 2000; Conte et al., 2004).

In the current study, vitamin E was shown to induce microglial proliferation with concomitant telomere shortening in vitro. However, we have found that telomeres lengthen in axotomy-activated proliferating rat microglia in vivo (Flanary and Streit, submitted). Although it remains unknown what effects, if any, vitamin E has on microglia in vivo, it remains possible that vitamin E supplementation in rodents/humans may also induce a similar high proliferative rate of microglia in vivo. If proliferation occurs, this may result in an accelerated rate of telomere shortening in microglia, in particular because all examined human somatic cell types do not express telomerase activity (Kim et al., 1994), and thus may hasten their entry into replicative senescence (Flanary, 2004; Fossel, 2004). However, telomerase activity has been detected in human lymphocytes and hematopoietic progenitor cells (Hiyama et al., 1995). Since human
microglia originate from bone marrow-derived hematopoietic progenitor cells (Eglitis and Mezey, 1997; Hess et al., 2004), they too may be able to upregulate telomerase activity in certain instances (and thereby possibly prevent/slow telomere shortening following periods of rapid division). The findings from this study provide an impetus to further investigate telomere biology in human microglia, as well as the roles of *in vivo* vitamin E supplementation in rats with age, and following CNS injury, in relation to telomere dynamics and microglial cell proliferation.
CHAPTER 6
LIFE-SPAN EXTENSION IN NORMAL RAT MICROGLIA VIA TELOMERE
REVERSE TRANSCRIPTASE RETROVIRAL TRANSDUCTION

Introduction

Telomeres in rat microglia (the only species examined to date) have been found to shorten with age when cultured \textit{in vitro} (Flanary and Streit, 2004). Thus, in order to determine whether exogenous delivery of the telomerase gene via retroviral transduction could prevent microglial senescence and extend the life-span of cultured rat microglia, we decided to measure parameters indicative of telomeric maintenance, such as telomere length and telomerase activity.

Materials and Methods

Culturing of Microglia

Microglia were isolated as described in Chapter 1.

Production of Replication-Defective Telomerase-Encoding Retroviruses

The retroviral vector, pLPC-hTRT, was obtained via a material transfer agreement (reference #: \(3354/MTA \text{ hTRT/Streit}\)) through Geron Corporation (Menlo Park, CA) and Clontech (Palo Alto, CA). The pLPC-hTRT retroviral vector expresses human telomerase reverse transcriptase (hTRT) from the cytomegalovirus promoter. The 5’ viral long terminal repeat controls expression of the transcript that contains \(\psi^{+}\) (the extended viral packaging signal), the puromycin resistance gene for antibiotic selection in eukaryotic cells, and telomerase. Vector DNA was amplified in \(\text{DH}5\alpha\) competent cells (Invitrogen, Carlsbad, CA, catalog #: \(18263-012\)) in the presence of ampicillin.
(resistance was conferred by the presence of pLPC-hTRT, which contained an ampicillin-resistance gene), purified using the hi-speed maxi prep kit (Qiagen, Valencia, CA, catalog #: 12662), and stored at -20°C until needed. DNA concentration was determined by absorbance at 260 nm, while DNA purity was calculated by the ratio of 260 nm versus 280 nm absorbance, using a spectrophotometer. The Phoenix amphotropic retroviral packaging cell line (a gift from Michel Ouellette, Ph.D., University of Nebraska Medical Center, Lincoln, NE) was used to produce telomerase-encoding retrovirus. Phoenix cells were thawed from cryovials stored in liquid nitrogen, expanded in DMEM, passaged via trypsinization, plated into 21 cm² dishes (2.5 to 5.0 x 10⁵ cells per dish), and allowed to divide overnight at 37°C under 5% CO₂. The following morning, cells that were 80 to 90% confluent were transfected with 10 to 20 μg of either: 1) pLPC-hTRT plasmid DNA, 2) empty vector (i.e., pLPC vector only), or 3) green fluorescence protein (GFP) vector (Clontech vector pLEGFP-C1, a gift from John Rossi at the Beckman Research Institute of the City of Hope, Duarte, CA) using the CaPO₄-mediated MBS mammalian transfection kit (Stratagene, La Jolla, CA) according to the manufacturers recommended transfection protocol. Retrovirus was harvested over the next 24 to 72 hours post-transfection by collecting the supernatant, and filter-sterilizing it using a 0.45 μm PVDF filter (Fisher Scientific, Pittsburgh, PA, catalog #: 09-720-4). Viral supernatants were either stored on ice and used immediately, or aliquoted and stored at -80°C (for up to 6 months) until used. Permission to use both the pLPC-hTRT retroviral vector and the replication-defective telomerase-encoding retroviruses was approved by the University of Florida Environmental Health and Safety office (project approval number: RD-2436).
Transduction of Rat Microglia With Telomerase-Encoding Retroviruses

Rat microglia, as well as a rat glioblastoma cell line (i.e., RG-2) were plated (on day 0) in 9.5 cm² plates (1.5 to 2.5 x 10⁵ cells/well) and allowed to divide overnight at 37°C under 5% CO₂. The following morning, media was aspirated, and retroviral transduction (using freshly-harvest retrovirus) was accomplished (on day 1 or 2) by adding a mixture containing 1 volume of viral supernatant, 1 volume of DMEM, and 4 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO, catalog #: H-9268) directly to the cells. Wells containing microglia also had 10.2 μM (0.15 μg/mL) of the mitogen recombinant rat granulocyte-macrophage colony stimulating factor (CSF) (R&D Systems, Minneapolis, MN, catalog #: 518-GM) added to the media to help stimulate cell proliferation (as required by retroviruses for transduction to occur). Infections performed included: 1) cells infected with pLPC-hTRT, 2) cells infected with pLPC empty vector, 3) cells infected with GFP vector (pLEGFP-C1A) or 4) cells infected with virus-free media (i.e., non-transduced). The pLPC-hTRT vector transiently expresses, or integrates and stably expresses, a transcript containing the extended viral packaging signal (ψ⁺), the puromycin resistance gene (PuroR), and human telomerase reverse transcriptase (hTRT). Only the protein component of telomerase was needed during transduction, since when the protein component binds with normal endogenous RNA subunits of telomerase, a functional enzyme will result. Transduction efficiency was monitored by transducing both rat microglia and RG-2 cells with a GFP vector (pLEGFP-C1, Clontech, Palo Alto, CA), and analyzing the cells for subsequent GFP expression using a Zeiss Axiovert 25 fluorescence inverted microscope. Within 24 to 72 hours, cells were infected sequentially with each of the different harvests of the same virus and
allowed to divide overnight (16 hours) at 37°C under 5% CO₂. Following transduction, CSF was added to microglia to stimulate cell proliferation and aid in the incorporation of the viral genome. Cells were selected for stable viral integrations using puromycin (Sigma-Aldrich, St. Louis, MO, catalog #: P-8833) at concentrations of 500 ng/mL for microglia. Puromycin was added to the media of all transduced cells (except for one set of cells that was not transduced at all) for approximately 7 days, or until all cells infected with virus-free media were dead. Transduced microglia were cultured in either the continual presence or absence of CSF. Cells were photographed at various time points using a digital camera (Sony DSC-S75 Cyber-shot, 3.3 megapixels, Carl Zeiss Vario-Sonnar lens) connected to a Zeiss Axiovert 25 fluorescence inverted microscope.

**Determination of Telomerase Activity**

Telomerase activity was measured as described in Chapter 1.

**Telomerase Western Blot Analysis**

Telomerase protein quantity was measured as described in Chapter 3.

**Statistical Analysis of Data**

Statistical analyses were performed as described in Chapter 3.

**Results**

**Telomerase-Encoding Retroviral Vector**

The map of the retroviral vector used in transfection and transduction experiments is shown in Fig. 6-1. The pLPC-hTRT retroviral vector contains elements derived from the Moloney murine leukemia virus, and expresses human telomerase reverse transcriptase (hTRT) driven from the cytomegalovirus promoter (CMV IE). The 5’ viral long terminal repeat (LTR) is the viral promoter that controls expression of the transcript containing ψ⁺, the viral packaging signal (required for encapsidation of the vector), the
puromycin resistance gene (Puro<sup>r</sup>), which is used when selecting mammalian cells post-
transduction, the telomerase gene (hTRT), and the 3’ viral LTR, which encodes the poly-
A signal. The ampicillin-resistance gene (Amp<sup>r</sup>), is used for propagation and selection in
bacteria, and the Col E1 is the origin of replication during bacterial propagation.

**Telomerase Transduction Extends Life-Span of Microglia**

Transduction efficiency was monitored by transducing both rat microglia and a rat
glioblastoma cell line (i.e., RG-2) with a GFP vector (pLEGFP-C1), and analyzing the
cells for subsequent GFP expression (Fig. 6-2). In both GFP-transduced microglia and
RG-2 cells, there was evidence that transduction was successful, since both cell types
exhibited numerous green fluorescent cells in the days immediately following
transduction. The efficiency of transduction was estimated to be approximately 10%.
Microglia transduced with telomerase lived longer than both controls and pLPC (non-
transduced) and empty-vector transduced microglia were all dead by day 20 (transduction
day = day 1) (Fig. 6-3, 6-4). However, microglia transduced with the telomerase-
encoding retrovirus were able to live until day 75, representing an increase in maximal
life-span of 375%. In a follow-up experiment, in which microglia were cultured in the
presence of CSF only during the first few days following transduction, both controls and
empty-vector transduced microglia were all dead by day 27. However, microglia
transduced with telomerase survived until day 62, representing an increase in maximal
life-span of 230%. In both experiments, telomerase-transduced microglia gradually
slowed in their cell division rate, and eventually stopped dividing around day 50.
Figure 6-1. The Clontech retroviral vector, pLPC-hTRT, used to transduce cultured rat microglia with the human telomerase reverse transcriptase (i.e., hTRT) gene. The 5’ viral long terminal repeat (LTR) contains promoter/enhancer sequences that control expression of the puromycin resistance gene (Puro$^R$) for antibiotic selection in eukaryotic cells. Expression of hTRT is driven by the human cytomegalovirus (CMV) immediate early promoter ($P_{CMV\,IE}$).
Figure 6-2. Brightfield and green fluorescence micrographs of rat microglia and rat glioblastoma cells (RG-2) following retroviral transduction on day 4. A: rat microglia brightfield image (arrow indicates GFP-positive cell); B: rat microglia fluorescence image (of A); C: RG-2 brightfield image (arrows indicate GFP-positive cells); D: RG-2 fluorescence image (of C). Note the GFP expressing microglia and RG-2 cells, indicating that transfection and subsequent transduction was successful. Scale bar = 20.0 μm.

Telomerase Activity in Transduced Microglia

TRAP analysis was used for determination of telomerase activity in transduced rat microglia (Fig. 6-5). Telomerase activity increased from day 0 to 2, and declined significantly (p<0.001) from day 2 to 6 in controls and hTRT-transduced microglia (Fig. 6-6). From day 6 onward, telomerase activity remained relatively unchanged in these two groups. In pLPC (empty vector)-transduced microglia, telomerase activity also
Figure 6-3. Representative micrographs of cultured rat microglia following retroviral transduction on days 1 and 20. A to C: Day 1; D to F: Day 20. A and D = non-transduced microglia (control); B and E = pLPC empty vector-transduced microglia; C and F = hTRT-transduced microglia. By day 20, both control and empty vector-transduced cells were all dead, whereas microglia transduced with hTRT were still alive on the same day. Scale bar = 20.0 μm.
Figure 6-4. Representative photographs of hTRT-transduced cultured rat microglia on days 57 and 75. A to C: Day 57; D to F: Day 75. By day 75, only a very small percentage of microglia transduced with hTRT were still alive. Scale bar = 20.0 μm.
declined significantly ($p<0.001$) from day 2 to 3, increased moderately from day 3 to 6, then declined significantly ($p<0.001$) from day 6 to 22. In this experiment, both controls and empty-vector transduced microglia were all dead by day 27, whereas hTRT-transduced microglia survived for 62 days and exhibited telomerase activity at least up to day 37.

Figure 6-5. TRAP analysis image used for measurement of telomerase activity of cultured rat microglia on the indicated days under various transduction conditions. Microglia were harvested on day 0, and transduced on days 2 and 4. Both controls and empty-vector transduced microglia were all dead by day 27, whereas hTRT-transduced microglia survived until day 62. Con = non-transduced; pLPC = empty vector (pLPC)-transduced, hTRT = telomerase-transduced; Neg = telomerase-negative control; Pos = telomerase-positive control; IC = internal control.
Figure 6-6. Quantitation of telomerase activity in cultured rat microglia on the indicated days under various transduction conditions. Con = non-transduced; pLPC = empty vector (pLPC)-transduced, hTRT = telomerase-transduced.

**Telomerase Protein Quantity in Telomerase-Transduced Microglia**

Western blotting was used for determination of telomerase protein quantity in transduced rat microglia (Fig. 6-7). Quantitation of telomerase protein is shown in Fig. 6-8. Telomerase protein increased from day 0 to 2, and gradually declined from day 2 to 22 in controls and empty vector-transduced microglia. In hTRT-transduced microglia, telomerase protein quantity declined significantly (p<0.001) from day 2 to 6, then slightly increased from day 6 to 37. In this experiment, both controls and empty-vector transduced microglia were all dead by day 27, whereas hTRT-transduced microglia survived for 62 days and still produced telomerase protein at least up to day 37.
Figure 6-7. Western blot image used for measurement of telomerase protein in cultured rat microglia on the indicated days under various transduction conditions. Microglia were harvested on day 0, and transduced on days 2 and 4. Both controls and empty-vector transduced microglia were all dead by day 27, whereas hTRT-transduced microglia survived until day 62. Con = non-transduced; pLPC = empty vector (pLPC)-transduced, hTRT = telomerase-transduced.

Figure 6-8. Quantitation of telomerase protein quantity in cultured rat microglia on the indicated days under various transduction conditions. Con = non-transduced; pLPC = empty vector (pLPC)-transduced, hTRT = telomerase-transduced.
Discussion

We have shown, for the first time, that exogenous delivery of the telomerase gene in normal rat microglia can extend their replicative and maximal life-span. In addition, telomerase-transduced microglia exhibited a delay in their entry to senescence, and maintained a normal phenotype following transduction. Our findings suggest that exogenous delivery of the telomerase gene can extend maximal life-span in cultured rat microglia.

Despite low efficiency, retroviral transduction was successful, as evidenced by GFP expression in both microglia and RG-2 cells. Transduction of rat microglia with catalytic component of telomerase (i.e., hTRT) resulted in life-span extension compared to controls (non-transduced) and empty-vector transduced cells. Telomerase activity/protein, albeit at low levels, were present in telomerase-transduced microglia well past the timepoint when control cells had all died. In addition, the pattern of telomerase activity and telomerase protein quantity corresponded well, suggesting that there is a synergistic effect between the two (as expected). There is an approximate 7-fold decrease in protein quantity (as determined by Western blot analysis) in telomerase-transduced microglia compared to controls. Importantly, a similar level of telomerase activity exists in all groups, suggesting that the low levels of telomerase protein present within transduced microglia are very active and have considerable catalytic activity.

Telomerase transduced microglia cultured in the continual presence of recombinant rat granulocyte macrophage-colony stimulating factor (CSF) lived 375% longer than controls. Telomerase transduced microglia cultured in the absence of CSF (except during the transduction process) lived 230% longer than controls. Previous research from our laboratory has shown that CSF-treated rat microglia exhibit an increase in both cell
proliferation and telomerase activity (Flanary and Streit, 2004). The transduced microglia cultured continuously with CSF may have lived longer (by about 20%) than those cultured in the absence of CSF due to the continual presence of the mitogen CSF, which may have acted to induce cell proliferation and thereby promote the retention of the transduced telomerase plasmid by the cells. In the absence of such mitogenic stimuli, the transduced cells may have lost the telomerase plasmid (or excised it from the genome if previously incorporated), since proliferation (and therefore an increase in telomerase activity) was not required. Interestingly, control cells lived only until day 20 when cultured in continual presence of CSF. However, when they were cultured in CSF for only the first few days, the control cells lived until day 27, representing a 35% increase in maximal life-span (compared to those that lived until day 20). When the control cells were cultured in the continual presence of CSF, it likely induced an increase in cell proliferation (i.e., at a higher rate compared when CSF was limiting), and hence telomere shortening, which may have hastened their entry into replicative senescence. Thus, control cells cultured in the presence of CSF for the first few days only were not induced to divide as much, and as a result, had a slower rate of telomere attrition which may have delayed their entry into replicative senescence. In addition, a parallel experiment (data not shown) was also conducted for rat astrocytes telomerase transduction. Both controls and empty-vector transduced astrocytes were all dead by day 44, however, astrocytes transduced with telomerase lived until day 134 (305% increase in maximal life-span).

Previous studies have shown that hTRT transduction can be used to significantly extend the replicative life-span of human cells, resulting in immortalization (Bodnar et al., 1998). In the present study, hTRT transduction of rat microglia did not result
immortalization, but rather only extended the life-span of these cells. Rat somatic cells normally express telomerase (Golubovskaya et al., 1997), and telomerase transduction to any rat cell type has never been reported previously, as nearly all previous experiments that delivered exogenous telomerase did so to human cell types. Similar to our results, previous studies in human myocytes (Di Donna et al., 2003) and human brain endothelial cells (HBEC’s) (Gu et al., 2003) have also found that telomerase transduction only results in life-span extension, but not immortalization. Interestingly, the maximum extension of life-span observed in human myocytes in the study by Di Donna et al. (2003) was 225%. Likewise, the maximal life-span extension of HBEC’s in the study by Gu et al. (2003) was 257%. Similarly, in the present study, we observed a maximal increase in cell life-span of 230% (in microglia cultured in the absence of continual CSF stimulation) and 375% (in microglia cultured in continual CSF stimulation). Why are rat microglia not immortalized, and only have an extension in life-span, following retroviral telomerase transduction? Perhaps the ability of exogenous telomerase to act on telomeres and extend cell life-span is affected by the promoter strength, site of integration within the genome of the host cell, levels of endogenous rTR (rat telomerase RNA component) and rTRT (rat telomerase reverse transcriptase) present, telomere and/or telomerase-associated proteins, and the levels of telomerase activity produced. A threshold level of telomerase activity may be required for immortalization, as previous studies have shown that transduced cells exhibiting low levels of telomerase are insufficient to prevent telomere shortening (Bodnar et al., 1998). In the present study, lack of immortality suggests that hTRT gene was not incorporated into the genome, but was rather only transiently expressed and was maintained as an extra-chromosomal plasmid which was eventually
lost/degraded over time. In addition, telomerase activity may seem apparent when total protein is isolated and measured following in vitro culturing, yet the enzyme could be inhibited by a repressor molecule while in vitro or in vivo, which may or may not be present within the total protein pool during analysis. Additional in vitro or in vivo molecules may also play critical roles in regulating telomerase activity. Thus, the pattern of telomerase activity, as determined by in vitro total protein analysis, may not correlate precisely with or imply telomere maintenance in vitro or in vivo (Ouellette et al., 1999).

The level of telomerase expression/activity required to adequately maintain telomere length in any cell type has not been determined, and thus different expression/activity patterns may exist when comparing in vitro analyses to the actual in vivo environment.

The role that microglial telomere shortening and senescence plays in normal brain aging and in AD is not understood. Cellular senescence in microglia has not been studied as a contributing factor in neuron and synapse loss during aging. Since microglia can normally clear Aβ (amyloid-beta peptide) (Frautschy et al., 1992), and are known to proliferate in the adult brain (and are thus susceptible to telomere shortening), they represent an attractive cell population for telomerase immortalization. Previous research in our laboratory has found that telomeres in rat microglia (the only species examined thus far) do shorten with age when cultured in vitro (Flanary and Streit, 2004). However, cells in vivo are exposed to a multitude of cell signals and effector molecules, as well as an extracellular environment that is currently impossible to replicate in an in vitro environment. Thus, while learning from in vitro experimentation will help take us take the research to the next level (in vivo), it will be this next level that holds the most promise for clinical intervention of any disease. Experiments using telomerase
activation/over-expression may pave the way for establishing an anti-aging therapy that can be applied to an aging in vivo multicellular system (i.e., humans). To date, all research published on telomerase transduction/immortalization in has been performed in vitro. Thus, while we may attain remarkable, even unprecedented, success in these in vitro experiments, the in vivo animal studies that are essential and will eventually follow may or may not show similar results. Therefore, placing an emphasis on proceeding to in vivo trials is a necessary “second step” that to date has yet to be taken. Additional experiments (both in vitro and ex vivo), as well as human clinical trials, are necessary in order to accurately confirm or deny whether telomerase induction/over-expression within microglia in vivo in a telomerase-negative multicellular organism can slow/prevent their telomere shortening and senescence, and as a result, slow/prevent the onset of AD.

If microglia undergo telomere shortening with age in vivo in the adult human brain, then this suggests that their subsequent entry into senescence also occurs. Indeed, future studies in our laboratory will examine telomere length and telomerase activity in human microglia isolated post-mortem from normal and AD-demented individuals. As senescent microglial cells amass, they may become dysfunctional and less able to sustain their neuron-supporting functions, ultimately leading to neuronal dysfunction and the eventual death of the neurons they once supported. Neuronal death results in a loss of synaptic connections, which ultimately is the causal factor of progressive memory loss with age. Thus, preventing telomere shortening (e.g., with exogenous telomerase delivery) in microglia may prevent their senescence and enable these cells to carry out their normal functions for a longer period of time. In vivo re-implantation of ex vivo-transduced rat microglia has been successfully performed previously (Mordelet et al.,
2002; Watanabe et al., 2002), supporting the notion that the same could hold true for humans in vivo. Perhaps extending the life-span of microglia by immortalizing them with telomerase could enable them to function normally for a longer period of time. This may enable these cells to adequately clear Aβ before it aggregates into neurotoxic plaques. If plaque formation can be prevented, then so might neuronal cell death and synapse loss. If synapses can be maintained within the brain for a longer period of time, then additional years or decades of critical thinking and memory retrieval may be possible. A concern is that expression of telomerase in normal somatic cells (e.g., microglia) may induce, or aid in the development of, tumorigenesis. However, other telomerase-transduced cell types have not resulted in the acquisition of tumorigenic properties (Belair et al., 1997; Jiang et al., 1999; Morales et al., 1999; Harley, 2002). In the current study, we have not observed any gross phenotypic or morphological characteristics (e.g., loss of contact inhibition), or anchorage-independent growth of transduced microglia that indicate tumorigenicity, or that may account for the extended cell life-span of telomerase-transduced microglia. A better understanding of the molecular mechanisms of microglial telomere biology could provide a novel perspective for understanding the development and pathogenesis of neurodegenerative diseases, and lead to the development of new drugs designed to enhance microglial cell function and/or to slow microglial telomere shortening (e.g., via a telomerase inducer molecule) and senescence as treatments of AD.
Collectively, these experiments have focused on studying the hypothesis that with aging, microglia undergo telomere shortening both \textit{in vitro} and \textit{in vivo}, become increasingly dysfunctional, and ultimately enter cellular senescence. The rationale for this hypothesis is based on the fact that microglia undergo cell division \textit{in vivo}, and are thus susceptible to telomere shortening with age. If this situation does indeed occur \textit{in vivo} in multicellular telomerase-negative organisms (e.g., humans), it may lead to a decline in microglial cell function with age, which in turn, would inhibit their ability to promote neuronal well-being. Thus, age-related neuron loss may be due to loss of microglial support.

Telomere shortening was found to occur in cultured rat microglia \textit{in vitro}, especially under periods of high proliferation (i.e., GM-CSF stimulation), and with additional cell divisions (i.e., with increasing culture area). Microglia undergoing continual rapid division \textit{in vitro} apparently “use up” the majority of their replicative potential prematurely, with the increased proliferation corresponding to both increased telomere attrition and earlier entry into senescence. An interesting future study would be to examine telomere length in acutely-isolated [i.e., via fluorescence-activated cell sorting (FACS)] microglia to determine if telomere shortening also occurs \textit{in vivo}, and at what rate.
Telomere shortening also occurred with age in rat cerebellum and cortex tissues \textit{in vivo}. However, since the tissue samples analyzed represented a mixed population of cell types (both glial and neuronal), it remained difficult to conclusively state that telomere shortening occurred in microglial cells specifically with age \textit{in vivo}. Thus, future experiments will likely focus on examining telomere dynamics in acutely-isolated (i.e., via FACS) microglia to determine if microglia are the cell type responsible for the decrease in telomere length evident with age. In addition, looking beyond 6 months of age will provide a more detailed understanding of the rate of shortening with age in specific brain tissue types. Perhaps one of the most important studies to be performed in this area is to examine human microglial cells and measure telomere length and telomerase activity to determine if they undergo telomere shortening with age and incidence of Alzheimer’s disease (AD). Coincidentally, our laboratory has currently received five cases (i.e., human microglial cell pellets) to date from the Sun Health Research Institute (Sun City, AZ), with additional cases being collected. This experiment, if successful in showing telomere loss with age and incidence of AD in human microglia, would provide the most support to our hypothesis that telomere shortening in microglia contributes to normal brain aging and memory loss.

Maintenance and extension of telomere length via the action of the telomerase enzyme was shown to occur in activated, proliferating microglia that accumulate in the axotomized rat facial motor nuclei. This result was somewhat surprising, since we hypothesized that with increased cell division \textit{in vivo} that microglia would undergo telomere shortening. However, the fact that telomerase activity was much higher in axotomized whole facial nuclei tissue suggests that the increase in telomere length was
due to a similar increase in activity. Interestingly, a similar situation occurred in vitro when microglia were stimulated to rapidly divide with GM-CSF, since this also caused dramatic telomere lengthening initially (followed by telomere attrition). It is unknown how telomere length would change at future time-points (i.e., more than four days) following axotomy, however, this will be the subject of future studies. In addition, since the increase in telomere length was shown to occur in whole facial nucleus tissues, and not in microglia directly, additional studies examining telomere length and telomerase activity at various time-points in acutely-isolated (i.e., via FACS) microglia following single and repeated axotomy are warranted to confirm that the increases evident in telomere length in whole facial nuclei tissues stem from an increase microglial telomere length. If repeated axotomy does cause telomere shortening in microglia, this would have implications and a correlation to repeated head injury cases in humans, and would suggest that head injuries could cause an increase in telomere shortening and senescence (due to increased division rate of microglia), leading to premature memory loss and dementia, which is known to occur in these individuals (e.g., football and soccer players, boxers).

Treatment of rat microglial cells with vitamin E caused dramatic microglial proliferation in vitro. This result was confirmed on numerous instances using different stocks/lots of vitamin E, as well as different cultures of rat microglia. The increase in proliferation evident also resulted in a concomitant decrease in telomere length and telomerase activity. Cultures treated in parallel with the antioxidant α-lipoic acid showed no proliferative response, indicating that the mitogenic effect of vitamin E is independent of its antioxidative action. Vitamin E-induced rat microglial proliferation was higher
than in cells stimulated with GM-CSF (a well-known and characterized mitogen),
suggesting that vitamin E is the most potent known microglial mitogen. Vitamin E-
treated microglia undergoing rapid division apparently “use up” the majority of their
replicative potential prematurely, as evidenced by an increased rate of telomere attrition
and earlier entry into cellular senescence, compared to controls. Future studies will
examine the effects of vitamin E supplementation to determine if telomere shortening
occurs in vivo. If so, this would have implications for the use of vitamin E as a dietary
supplement. While vitamin E is clearly beneficial for health as determined by a number
of studies and clinical trials, and irrelevant of whether telomere shortening occurs in vivo
in vitamin E-supplemented rats (especially since rats normally express telomerase
activity in their microglia), if vitamin E causes proliferation of microglia in humans, this
may contribute to an advanced rate of telomere shortening, early entrance into
senescence, and premature development of age-related memory loss (if senescent
microglia are truly a contributing factor).

Retroviral delivery of the protein component of telomerase appears to extend cell
life-span and delay senescence in both rat microglia and astrocytes. However, only a
GFP vector was shown to have been successfully transduced in parallel experiments,
which suggests the successful transduction of the telomerase vector, but begs the question
of whether or not the telomerase vector actually entered the cells. Current studies in our
laboratory are attempting to answer this very question using PCR-based methodologies to
detect the presence of the vector DNA and relative copy number of the telomerase gene).

**Implications**

If telomere shortening occurs in human microglia with age and incidence of head
injury and dementia, then it seems reasonable that re-lengthening telomeres and/or
preventing further telomere erosion may prove beneficial as a potential treatment to the
dysfunction and senescence that may normally with age in microglia. If telomere
shortening occurs in human microglia \textit{in vivo}, this may lead to a decline in cell function
with age and inhibit their ability promote neuronal well-being. Thus, understanding the
molecular mechanisms of microglial telomere biology, how and why telomere shortening
triggers cellular senescence, and how microglia are involved in age-related deterioration
of neuronal function could provide a novel perspective to further understand the normal
aging process in microglia as well as the origins of CNS pathogenesis. This
understanding could lead to the development of new drugs designed to enhance
microglial cell function and/or slow microglial telomere shortening and senescence as
potential treatments for AD in humans. One possible therapy may be to induce
telomerase expression/activity, either by activating endogenous telomerase via removal
of repressor protein(s), or by the delivery of biologically-active and functional telomerase
enzyme directly to cells (especially those that are mitotically-active). This treatment may
slow/prevent telomere shortening and senescence in microglia and enable these cells to
promote neuronal well-being and perform other vital functions (e.g., clearing amyloid)
for a longer time period, which may permit additional years of critical thinking ability
and memory retrieval, and slow/prevent the onset of pathological conditions involving
senescent microglia
LIST OF REFERENCES


di Donna S, Mamchaoui K, Cooper RN, Seigneurin-Venin S, Tremblay J, Butler-Browne GS, Moully V. 2003. Telomerase can extend the proliferative capacity of human myoblasts, but does not lead to their immortalization. Mol Cancer Res. 1, 643-653.


Flanary BE, Streit WJ.  Axotomy increases telomere length, telomerase activity and protein in axotomy-activated microglia.  Submitted for review to Neurobiol Aging.


Morin GB. 1989. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell. 59, 521-529.


Streit WJ. 2002b. Microglia as neuroprotective, immunocompetent cells of the CNS. Glia. 40, 133-139.


Wright WE, Brasiskyte D, Piatyszek MA, Shay JW. 1996a. Experimental elongation of telomeres extends the lifespan of immortal x normal cell hybrids. EMBO J. 15, 1734-1741.


BIOGRAPHICAL SKETCH

Barry Eric Flanary began pursuing his Doctor of Philosophy (Ph.D.) degree in biomedical sciences at the University of Florida (UFL) College of Medicine Department of Neuroscience in Gainesville in August of 2001. He received his Associate of Science (A.S.) degree in 1996 from Illinois Valley Community College, and his Bachelor of Science (B.S.) (1999) and Master of Science (M.S.) (2001) degrees in biological sciences from Illinois State University (ISU). Since 2000, he has served on the Editorial Board and written gerontology literature and book reviews for the *Journal of Anti-Aging Medicine*, the only peer-reviewed scientific journal dedicated to publishing research on altering clinical aging and age-related diseases. He is currently a member of the American Aging Association, the Gerontological Society of America, and the Phi Sigma Biological honor society. During work on his A.S. degree, he received two scholarships, and while working towards his B.S. degree, he was an undergraduate research fellow in a National Science Foundation research training program (collaborative research at undergraduate institutions) under a grant received by ISU. While working on his M.S. thesis, “Molecular Cloning, Characterization, and Mutagenesis of the msbB Gene, a Secondary Lipid A Acyltransferase, in *Haemophilus parainfluenzae*,” he received one teaching fellowship, one research fellowship, and two graduate student association research grants. He also served as a Phi Sigma biological honor society grant review committee member and as a graduate teaching assistant in introductory biology for one year, and introductory microbiology for one semester at ISU. From 1999 to 2001, he had
ongoing involvement as chief scientific advisor for a telomere-based art project (featured in the Siggraph 2001 art gallery) at Art to the Nth Power Inc. (www.artn.com/telomeres), a collaborative art group and media lab based in Chicago, IL. During work on his Ph.D. dissertation, “Analysis of Rat Microglial Cellular Senescence as Determined by Measurements of Telomere Length and Telomerase Activity,” he was competitively selected for and attended a National Institute on Aging Technical Assistance Workshop for Emerging Scientists and Students Seeking Careers in Aging Research in Boston, MA, twice served as a graduate student mentor, co-authored three research grants received from the Evelyn F. McKnight Brain Research Foundation, co-authored one R01 research grant received from the National Institute on Aging, received two graduate student council grants, one department of Neuroscience grant, one research fellowship, one graduate fellowship for outstanding research, three fellowships from the American Foundation for Aging Research, one fellowship from the Neurobiology of Aging program, one Science program for excellence in science award from the American Association for the Advancement of Science, two medical guild research incentive awards, and three endowments from the Bryan W. Robinson Memorial Endowment for the Neurosciences of the Tallahassee Memorial Hospital Foundation, Inc., at Florida State University. He has presented both his undergraduate and graduate research at numerous local, regional, national, and international research symposia since 1997. In March of 2004, he presented his research in a platform presentation as a plenary panel member on cellular aging and clinical interventions at the Inaugural (first) International Conference on Longevity in Sydney, Australia. His Ph.D. dissertation research focuses on telomere dynamics and cellular senescence in microglia. To date (March, 2005), his
publications include five first-author original research articles (3 are published, 2 are submitted for publication), 21 literature and book reviews, 14 abstracts, one book chapter, and one poem. While working on his Ph.D. dissertation, he married his beautiful wife, Allison.