Evidence that Aging and Amyloid Promote Microglial Cell Senescence

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ABSTRACT

Advanced age and presence of intracerebral amyloid deposits are known to be major risk factors for development of neurodegeneration in Alzheimer's disease (AD), and both have been associated with microglial activation. However, the specific role of activated microglia in AD pathogenesis remains unresolved. Here we report that microglial cells exhibit significant telomere shortening and reduction of telomerase activity with normal aging in rats, and that in humans there is a tendency toward telomere shortening with presence of dementia. Human brains containing high amyloid loads demonstrate a significantly higher degree of microglial dystrophy than nondemented, amyloid-free control subjects. Collectively, these findings show that microglial cell senescence associated with telomere shortening and normal aging is exacerbated by the presence of amyloid. They suggest that degeneration of microglia is a factor in the pathogenesis of AD.

INTRODUCTION

MICROGLIA REPRESENT A MAJOR glial cell population within the central nervous system (CNS) constituting the brain’s endogenous system of immunocompetent cells.1,2 Microglial cells are activated quickly in acute CNS injury situations when they undergo mitosis to increase their numbers.3,4 This acute neuroinflammatory response, which correlates well with the postaxotomy recovery of motoneurons and subsequent axonal regeneration, supports the view that microglia are constitutively neuroprotective cells.5,6 On the other hand, potentially detrimental, chronic microglial neuroinflammation and neurotoxicity have often been discussed as factors in the pathogenesis of neurodegenerative disorders, particularly Alzheimer’s disease (AD) where intracerebral amyloid deposits are thought to induce and sustain prolonged microglial activation accompanied by the production of microglial neurotoxins.7–11 However, postmortem observations have produced evidence showing that some of the ostensibly activated microglia in aged and diseased human brain also exhibit signs of cell death and cytoplasmic degeneration,12–16 the latter having been termed microglial dystrophy.12 These observations document that microglia themselves are subject to structural de-
terioration, and they raise the possibility that microglial degeneration may precede neurodegeneration and therefore contribute to it through diminishing neuroprotection.

Telomeres, the physical ends of eukaryotic chromosomes, naturally shorten during each cell division because of the inability of DNA polymerase to completely replicate linear DNA molecules. Certain cells can maintain their telomeres utilizing telomerase, an enzyme capable of relengthening telomeres. However, in somatic cells, telomeres continually shorten with age and division, and cells eventually exhaust their replicative potential, become incapable of further division, and ultimately enter replicative senescence, which is a nondividing state characterized by critically short telomeres, and substantial changes in cell function and gene expression. The shortest telomere length, not the average, is thought to be responsible for maintaining chromosome stability, cell viability, and determining when a cell will enter senescence. Recent work from this laboratory examining rat microglia telomeres and telomerase activity has shown that telomere shortening occurs over time in vitro and that mechanisms of telomere maintenance are active during microglial activation and proliferation in vivo supporting the idea that microglia, as the only mature cell type in the CNS capable of significant cell division, are subject to replicative senescence. In the current study, we have sought more direct evidence in support of microglial cell senescence focusing to incorporate the role of amyloid protein in this process. The findings reported here provide strong support for a novel view on AD pathogenesis that takes into account both aging and amyloid as important factors in a common pathway that involves progressive damage to the brain’s immune system.

MATERIALS AND METHODS

FACS isolation of rat microglia

Male Fisher-344 Brown Norway hybrid rats were exsanguinated while they were under deep sodium pentobarbital anesthesia (50 mg/kg body weight) using ice-cold phosphate-buffered saline (PBS). The brains were removed and placed in PBS on ice. Dissection and isolation of cerebral cortex from the chilled brains was performed immediately after removal. Cortex tissue was collected from 3- and 30-month-old rats (three animals per group). Tissue was processed according to established isolation protocols. Fluorescence-activated cell sort (FACS) analysis was performed with a FACS Vantage SE cell sorter and CellQuest software (BD Biosciences/Becton Dickinson, San Jose, CA). Monoclonal antibodies used to isolate microglia during FACS analysis were fluorescein isothiocyanate (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.

Culturing of rat microglia

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 × 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 g/mL Fungizone [Gibco, Carlsbad, CA], and 1 × 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 minutes at 37°C on a bidirectional tilting platform. An equal volume of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco) and 1% 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 minutes at 37°C on a bidirectional tilting platform. An equal volume of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco) 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, Briarcliff Manor, NY) and centrifuged (4000 rpm [2,900g], 10 minutes). 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 (4000 rpm [2900 g], 10 minutes), 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 × 10^6 cells per well), 3.8 cm² plates (4.0 × 10^5 cells per well), or 0.32 cm² plates (3.4 × 10^4 cells per 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 nonadherent cells. Microglia were then treated with the appropriate concentration of a particular treatment regimen.

Isolation of human microglia

Microglia were isolated from rapid autopsies of human brains as previously described. Briefly, brain tissue (approximately 50 g) for culture was taken from the superior frontal gyrus/frontal pole region. Tissue was minced with razor blades into small fragments and digested at 37°C with 0.15% trypsin (Invitrogen) in Hanks balanced salt solution and 1.0 mM ethylenediaminetetraacetic acid (EDTA). After 30 minutes of incubation, the tissue was triturated to break up fragments and DNAse was added to final concentration of 50 µg/mL. After an additional 30-minute incubation, the tissue was filtered through 100-µm nylon mesh and the digested material was spun at 800g for 10 minutes. Brain extracts were resuspended in serum-free DMEM and mixed with 90% (isotonic) Percoll (final concentration 30%). Extracts were centrifuged at 15,000g for 30 minutes. The top layer (myelin) was discarded and the Percoll layer was collected down to (not including) the red blood cell layer. Cells collected within this layer were washed twice in DMEM. The final cell pellet was resuspended in DMEM containing 10% FBS and cell number/viability was estimated using trypan blue staining. Cells were plated at a density of 50 million cells per T75 flask. Microglia attach rapidly to uncoated plastic while other cells in this suspension (i.e., oligodendrocytes and astrocytes) do not. Following a 12-hour incubation at 37°C, the nonadherent cells were removed and the adherent cells rinsed and resuspended with DMEM containing 10% FBS. Microglia were cultured for 10 to 14 days without media change. Approximately 500,000 microglia per case were collected from the culture surface using 0.25% trypsin per 1.0 mM EDTA, centrifuged to a pellet, and stored at −80°C.

Human brain samples

All tissue specimens used in this study were obtained from the brain bank at the Sun Health Research Institute, Sun City, Arizona. Tissue samples of frontal and temporal cortical regions that had been fixed in 4% paraformaldehyde solution for 48 hours were shipped in glycol storage solution and processed for immunohistochemistry.

Determination of telomerase activity by TRAP assay

Telomerase activity was measured using the telomere repeat amplification protocol (TRAP). Total protein was isolated from (1) FACS-isolated rat microglia, (2) isolated human microglia, and (3) cultured rat microglia using 200 µL CHAPS lysis buffer (5.0 mM β-mercaptoethanol, 1.0 mM ethyleneglycoltetraacetic acid [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 minutes, and centrifuged at 12,000g (14,000 rpm) for 20 minutes 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) according to 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 minutes at room temperature), 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 10× TRAP buffer (10 mM EGTA, 500 mM KCl, 15 mM MgCl₂, 100 mM Tris, 1.0 mg/mL bovine serum albumin [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 minutes 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-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 ladder of products (generated by telomerase) containing 6-base increments beginning at 40 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. PCR was carried out as follows: Initial denaturation at 94°C for 2 minutes to inactivate telomerase, then 33 total cycles of the following: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds. 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% nondenaturing polyacrylamide gel, and electrophoresed at 87 volts at room temperature in 0.5× 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 minutes 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).

Determination of telomere length by flow-FISH

Telomere length was measured using flow cytometry fluorescent in situ hybridization (flow-FISH). Briefly, 5 × 10⁵ cells were washed in PBS and resuspended in hybridization mixture (70% deionized formamide, 20 mM Tris [pH 7.0], 0.1% BSA, 0.3 μg/mL telomere-specific FITC-conjugated [C₃TA₂]₃ peptide nucleic acid [PNA] probe; Applied Biosystems, Foster City, CA; catalog # EX-030325-PP). For negative controls, cells were resuspended in hybridization mixture without the PNA probe. Samples were heat-denatured at 80°C for 10 minutes, followed by incubation in dark at room temperature for 90 minutes with gentle agitation. Samples were centrifuged and washed in 500 μL wash buffer I (70% formamide, 10 mM Tris, 0.1% BSA) for 2.5 minutes at room temperature, followed by 500 μL wash buffer II (5.0% glucose, 10 mM HEPES, 1.0% BSA, 1.0% Tween-20) for 1.5 minutes at room temperature. Cells were resuspended at a density of 1 × 10⁵ cells per 100 μL in PBS containing 0.1% BSA and 10 μg/mL RNase A. Flow cytometric analysis was performed using a FACSort flow cytometer (BD Biosciences, San Jose, CA) and CellQuest software (version 3.3). Measured fluorescent intensity was converted using the quantum 26 premixed quantitative standards kit (Bangs Laboratories, Fishers, IN; catalog # 826-P) to determine molecules of equivalent soluble fluorochrome (MESF) units.

Tissue processing and immunohistochemical staining

Tissue blocks were embedded in agarose and sections were cut on a vibratome at 50 μM thickness. The sections were incubated in blocking buffer consisting of PBS, pH 7.4, and
10% normal goat serum (NGS) for 1 hour at room temperature. Primary mouse monoclonal antibodies, LN-3 (MP Biomedicals, Irvine, CA) against human HLA-DR and 6F/3D (Dako, Carpinteria, CA) against human amyloid beta protein (Aβ8–17) were diluted 1:500 and 1:100, respectively, in PBS containing 5% NGS and sections were incubated overnight at 4°C. Sections were then rinsed three times with PBS, followed by incubation with biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA) diluted 1:500 in PBS and 5% NGS for 1 hour at room temperature. Sections were rinsed three times in PBS, and binding sites were visualized using horseradish peroxidase (HRP)-avidin D (Vector Laboratories) at 1:500 for 30 minutes followed by diaminobenzidine-H2O2 substrate medium. Sections were dehydrated through ascending ethanols, cleared in xylene, and coverslipped with Permount (Fisher Scientific). Selected sections were counterstained using cresyl violet. All preparations were viewed and photographed in a Zeiss Axiosplan photomicroscope.

Morphometric quantitation

Qualitative screening of LN-3–stained preparations identified six categories of microglial abnormalities: Clusters of cells, cytoplasmic fragmentations, deramifications, spheroids, atrophy, and beading. A quantitative measure of the incidences (count/mm²) of dystrophic microglia was performed using Image Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD), Spot camera and Spot Advanced software, and a Zeiss Axioskop 2 Plus with a 40× objective. Manual counts of microglial abnormalities were conducted by a blinded observer on 20 randomly selected fields of view (225 × 300 μM) of cortical gray matter for each case. One-way analysis of variance (ANOVA) followed by Tukey analyses (when appropriate) were conducted for all cell morphologies examined.

Statistical analysis of data

All data were analyzed using the software program InStat version 3.06 (GraphPad, San Diego, CA).

RESULTS

Telomere length shortens in rat microglia with age in vivo

As a first step in our investigation, we acutely isolated microglia from both young (3-month-old) and aged (30-month-old) rat cortical tissue using flow cytometric sorting. Isolated cells were then analyzed for telomere length and telomerase activity using telomere flow-FISH and telomerase repeat amplification protocol (TRAP), respectively. Telomere flow-FISH analysis of specific intervals within the total distribution curve (Fig. 1A) revealed that aged rats possessed significantly shorter microglia telomeres in all instances compared to young animals (Fig. 1B), despite only having a small number of total samples to analyze. The telomeres in the M2 population of microglia from three MO rats were significantly longer, and represent a significantly higher number of total flow events, compared to 30 MO microglia (Fig. 1B and 1C).

A concomitant significant decrease (p = 0.038) in telomerase activity was also evident in aged rats compared to young animals (Fig. 1D). Collectively, these findings provide in vivo confirmation of prior in vitro studies25 that demonstrated that rat microglial cells undergo telomere shortening with normal aging, and are thus subject to replicative senescence.

Telomere length analysis in human microglia in vivo with progression of AD

To address the subject of microglia telomere length and telomerase activity in human age-related neurodegenerative disease, microglial cells were isolated and cultured from the cerebral cortices of four individuals with significant AD dementia (Braak stage III or greater) and one nondemented individual (Table 1). DNA and protein were extracted from frozen cell pellets and subjected to telomere length and telomerase activity analyses using telomere flow-FISH and TRAP assay, respectively. The longest telomeres were found in the nondemented subject while all four individuals with AD pathology possessed shorter microglia telomeres (Fig. 2A and 2B), as evidenced by quantification of FACS analysis profiles and as-
FIG. 1. Telomere length significantly shortens in rat microglia with age \textit{in vivo}. Microglia were acutely isolated via fluorescence-activated cell sorting (FACS) from 3- or 30-month-old (MO) rat cortical tissue. Telomere length (i.e., MESF: molecules of equivalent soluble fluorochrome) was measured in FACS-isolated microglia using flow cytometry. Representative telomere length distributions from a 3- or 30-month-old rat, and the two subpopulations of lengths examined therein (i.e., M1, M2), in FACS-isolated microglia from 3- or 30-month-old rats (A). Microglia from 30 MO rats had significantly shorter telomere lengths (mean ± standard deviation [SD]), as shown by: a left-shifted curve (A), which indicates shorter telomeres, and graphically (B) in each of two separate areas examined. Three MO rats had a separate, smaller subpopulation of microglia (M2) that possessed very long telomeres that were absent in microglia from 30 MO rats (A). The telomeres in the M2 population of microglia from 3 MO rats were significantly longer (mean ± SD), and represent a significantly higher number of total flow events (mean ± SD) (i.e., total cells), compared to 30 MO microglia (B, C). Statistics were performed on all samples (n = 3) using a two-tailed, unpaired \( t \) test. Telomerase activity significantly declined in rat microglia with age \textit{in vivo}. Telomerase activity was measured using the telomere repeat amplification protocol (TRAP) assay. Representative telomerase activity distributions in FACS-isolated microglia from 3- or 30-month-old rats (D). Microglia from 30 MO rats had significantly less telomerase activity (mean ± SD). Neg, telomerase-negative control; Pos, telomerase-positive control. Statistics were performed on all samples (n = 3) using a one-tailed, unpaired \( t \) test.
assessment of the 95% confidence level of the distribution for the four AD cases (i.e., the nondemented case is greater than 2 standard deviations away from the mean of the four AD cases). In addition, short telomeres (i.e., fluorescent intensity values under approximately 8.0) were virtually absent in the nondemented individual, whereas all four AD cases clearly exhibited a higher quantity of short telomeres, as well as a smaller quantity of longer telomeres. Telomeres were longer than expected in the most advanced case (number 5; Braak stage VI), however this was also the youngest subject studied. A clear delineation between telomere length and progression of AD was not distinguishable in this group of subjects. Collectively, AD individuals had a larger quantity of short telomeres compared to the nondemented (ND) individual, as evidenced by the fact that the percent total events of the shortest telomeres (M1 population) of microglia from the ND individual were more than two standard deviations away from those of the AD group (Fig. 2C). Telomerase activity remained relatively unchanged among subjects studied (Fig. 2D), and does not provide independent evidence supporting the current hypothesis.

**Dystrophic microglia increase in AD individuals**

To further address the issue of pathogenesis in a manner that takes into account the presence of amyloid, we decided to use the incidence of microglial dystrophy as a different measure of cell senescence and perform histomorphometric studies. We previously reported an increased incidence of dystrophic microglia in human brain with normal aging in amyloid-free subjects, and were thus interested in determining whether the presence of amyloid could be correlated with an escalation of microglial dystrophy. Brain sections were analyzed from the superior frontal and temporal gyri of subjects classified into three categories based on mental status exam and on postmortem histopathology (Table 2). They were: (1) ND and amyloid-free individuals, (2) nondemented individuals with a high amyloid burden, designated as high pathology controls (HPC), and (3) demented individuals exhibiting classic Alzheimer-type pathology (AD) with high amyloid load. Dystrophic microglia were defined as cells clustered at sites of amyloid deposition, as well as cells displaying cytoplasmic abnormalities, such as bulbous spheroids, loss of fine processes (deramification), beading, and fragmentation of processes. Microglia were visualized immunohistochemically using LN-3 antibody directed at human HLA-DR antigens; amyloid was detected immunohistochemically in adjacent sections. Microscopic comparisons of adjacent sections stained for microglia and for amyloid β (Aβ) revealed a conspicuous presence of dystrophic microglia in AD and HPC subjects (even at low magnification), while cells in ND subjects displayed a more normal, ramified phenotype characteristic of so-called resting microglia (Fig. 3A–3F). The presence of dystrophic microglia was not limited to sites of amyloid deposition, as the cells were found singly and seemingly at random amongst all HLA-DR–positive cells. In many instances, several of the dystrophic changes were seen to occur simultaneously in the same microglial cell (Fig. 4A–4D). For example, some deramified pro-

<table>
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Subjects were classified into categories based on mental status exam and post-mortem histopathology. ND, nondemented; AD, Alzheimer’s disease.

**Table 1. Data of human subjects used for microglial isolation**
**A**

Fluorescent Intensity FL-1 PNA-FITC

**B**

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<th>M(ESF (Telomere Length)</th>
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**C**

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<tr>
<td>AD</td>
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**D**

Average Telomerase Activity

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<th>4</th>
<th>5</th>
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</table>
cesses also showed spheroids and/or beading, and some of the atrophic cytoplasmic processes showed swellings that could have been classified as either beads or spheroids. Morphometric quantification revealed a significant increase in the average incidence of dystrophic microglia per group in both HPC ($p = 0.038$) and AD ($p = 0.046$) individuals, relative to ND subjects (Fig. 4E), as well as in HPC plus AD compared to ND ($p = 0.031$; Fig. 4F). Interestingly, the average incidence was slightly higher in HPC individuals, a finding that supports the observation that there is no consistent direct correlation between the number of senile plaques and the severity of AD dementia.

**DISCUSSION**

Using acutely isolated microglia from rats, we have established that telomere shortening in microglia occurs during normal brain aging. The results support our hypothesis that microglia are subject to replicative senescence. Interestingly, microglia isolated from 3-month-old rats, but not 30-month-old rats, exhibited a separate subpopulation of microglia with very long telomeres. The absence of this cell population in aged animals suggests that these cells have undergone rapid cell division, resulting in their concomitant telomere shortening and the subsequent disappearance. While it is unknown what the function of this subpopulation of cells is, they could represent a small group of progenitor cells involved in reconstituting the microglial population in the young CNS. Future studies may examine additional age groups to identify a specific age range during which this cell population completely disappears, as well as perform functional studies of isolated cells within this population.

The current study is the first to report measurement of telomere length and telomerase activity in isolated human microglia. The results obtained from the human microglia telomere length studies provide an interesting glimpse into the realm of telomere biology and AD pathogenesis. Interestingly, short telomeres were virtually absent in the one nondemented subject, while all four AD cases clearly exhibited a higher quantity of short telomeres. Collectively, AD individuals not only had shorter overall telomere lengths compared to the ND individual, but they also had a larger quantity of short telomeres compared to the ND individual. Because senescence is triggered once any telomere in a cell reaches a critically-short length, these data suggest that microglia within AD individuals underwent replicative senescence earlier compared to the microglia within the age-matched nondemented individual. As microglia in AD individuals underwent telomere shortening and entered senescence, they may have been less able, or unable, to (1)

<table>
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<th>Case number</th>
<th>Age</th>
<th>Gender</th>
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ND, nondemented; AD, Alzheimer’s disease; HPC, high pathology control.
maintain neuronal health and (2) effectively phagocytose amyloid plaques. However, in addition to the confounding effects of aging, the human studies are associated with other caveats, namely, concerns regarding the maintenance of isolated cells for 10–14 days in vitro prior to telomere length and telomerase activity analyses. Culturing of human microglia following postmortem isolation is a necessary and unavoidable part of the harvesting process. While these human data do not support definitive conclusions regarding a link between microglial replicative senescence and neurodegeneration, they are (despite having a small number of total samples) nonetheless consistent with the hypotheses that microglia telomere shortening accelerates with progression of AD, and that the resultant senescent microglia may play a role in AD pathogenesis. However, measured telomerase activity in human microglia does not provide independent evidence supporting the current hypothesis.

The only prior study to examine telomere dynamics directly within the context of AD found significant telomere shortening and decreased telomerase activity in peripheral blood mononuclear cells in AD patients relative to controls, and that telomere length of T cells correlated with AD disease status, suggesting that AD directly/indirectly affects peripheral immune system cells. The data from the current study provide an interesting parallel in that telomere length in endogenous immune cells of the CNS is also shorter in AD individuals, and that this may be caused by amyloid plaque-induced proliferation.

The in situ evidence of microglial dystrophy in human brain provides additional support for a detrimental action of amyloid on microglia. Overall, the morphometric findings strongly support the hypothesis that presence of intracerebral amyloid adversely affects microglial cytoplasmic integrity, which may aggravate ongoing aging-related deterioration of the cells. In fact, prior studies conducted in vitro have shown that when cultured rat microglia are exposed to Aβ peptides, the cells show morphologic evidence of degeneration, such as bead-
ing of cytoplasmic processes and shrinkage of the cell body, changes that closely resemble the present observations in human brain.

Given that both aging and presence of amyloid are known to be major risk factors for development of AD, our findings bring to light a common denominator in these factors insofar as both may act as promoters of microglial degeneration. Because microglia are essential for maintaining neurons in the normal and, particularly, the injured/diseased CNS, and are also involved in the clearance of amyloid deposits, a deterioration of microglial function over a human lifetime could contribute to the development of neurofibrillary degeneration through waning glial neuroprotection, and to the formation of amyloid deposits through impaired clearance. This idea that aging and amyloid act synergistically to produce degenerative changes in the CNS is supported by studies showing that amyloid-mediated neurotoxicity is greater in aged than in young mon-
Our findings confirm and extend prior clues that insoluble amyloid aggregates adversely affect microglial cell function and structural integrity\textsuperscript{33,36,37} by providing a mechanism that involves microglial replicative senescence and dystrophy. As the only mature cell of the CNS with significant mitotic ability,\textsuperscript{5} the replicative potential of microglia may become exhausted with aging, and the decline in microglial renewal capacity may be further diminished by, and contribute to, the presence of amyloid, which may accelerate the process of telomere shortening. This finding has direct relevance for therapeutic strategies geared towards promoting microglial amyloid clearance,\textsuperscript{37–39} in that the success of vaccination-based therapies in aged humans may be limited by microglial cell senescence. The current findings do not support a direct connection between microglial degeneration and neurofibrillary degeneration, the factor most closely linked to dementia. The possibility remains that neurofibrillary degeneration may develop through an amyloid-independent mechanism.

Because microglia are capable of producing both neuroprotective and neurotoxic molecules, depending on the type of signals received from neurons\textsuperscript{40} 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 (1) maintain neuronal health and (2) effectively phagocytose amyloid plaques. 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, due to diminished glial support and maintenance. Neuronal cell death leads to loss of communication and synapses between neighboring neurons, which 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, which may inadvertently contribute to neurodegeneration (e.g., via decreased neuronal maintenance and plaque clearance) due to impaired glial support. Decreased plaque clearance would lead to an increase in amyloid plaques and, if plaques are mitogenic \textit{in vivo}, may accelerate microglia telomere shortening due to their increased division. Neuronal cell death is a hallmark characteristic of AD and may be linked to an impairment of microglial cell function. A better understanding of the molecular mechanisms of microglial telomere biology, how and why telomere shortening triggers cellular senescence, and how declining microglial function leads to age-related deterioration of neuronal function would provide an innovative perspective to further understand the normal aging process in microglia as well as the origins of CNS pathogenesis. 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. Strategies designed to enhance microglial cell function and/or to slow or prevent microglia telomere shortening may provide novel approaches toward AD prevention and treatment.\textsuperscript{41}

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