Microvasculature of the Mouse Cerebral Cortex Exhibits Increased Accumulation and Synthesis of Hyaluronan With Aging

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Abstract

The microvasculature of the aged brain is less dense and more vulnerable to dysfunction than that of the young brain. Brain microvasculature is supported by its surrounding extracellular matrix, which is comprised largely of hyaluronan (HA). HA is continually degraded into lower molecular weight forms that induce neuroinflammation. We examined HA associated with microvessels (MV) of the cerebral cortex of young (4 months), middle-aged (14 months), and aged (24–26 months) mice. We confirmed that the density of cortical MV decreased with age. Perivascular HA levels increased with age, but there was no age-associated change in HA molecular weight profile. MV isolated from aged cortex had more HA than MV from young cortex. Examination of mechanisms that might account for elevated HA levels with aging showed increased HA synthase 2 (HAS2) mRNA and protein in aged MV relative to young MV. In contrast, mRNAs for HA-degrading hyaluronidas or hyaladherins that mitigate HA degradation showed no changes with age. Corresponding to increased HAS2, aged MV synthesized significantly more HA (of all molecular weight classes) in vitro than young MV. We propose that increased HA synthesis and accumulation in brain MV contributes to neuroinflammation and reduced MV density and function in aging.

Keywords: Aging—Brain—Microvasculature—Hyaluronan—Microvessels
(<50 kDa) promotes inflammatory processes in the brain and other organs (14–16).

Little is known about the consequences of normal aging on HA in the brain of experimental animals (i.e., rodents) or humans. Utilizing electrophoretic separation of glycosaminoglycans, a substantial increase in HA levels relative to chondroitin sulfate was found in the brain tissue of 30-month-old rats compared to tissues from younger rats ranging from 10 days to 18 months of age (17). More recently, HA content, as measured by a highly sensitive enzyme-linked immunosorbent assay (ELISA), has been reported to increase with normal aging in nonhuman primates (macaques), especially in general brain gray matter (18). HA accumulation in the brain occurs as a result of all three HASes, but primarily reflects the activity of HAS1 and HAS2 (18–20).

The present study focuses on expression of HA in the microvasculature of the aging mouse brain. The mouse is an accepted model for studying normal aging of the brain and examining clinically relevant changes in the ECM (21,22). Herein, we examine the effects of aging on accumulation, synthesis and degradation of HA in the microvasculature of the cerebral cortex of young, middle-aged, and aged mice.

Materials and Methods

Animals

Young (4 months), middle-aged (14 months), and aged (24–26 months) male mice of the C57BL/6 strain were obtained from the National Institute of Aging Rodent Colony at Charles River Laboratories (Wilmington, MA). Mice were acclimated for 5 days prior to euthanasia. The Office of Animal Welfare at the University of Washington approved the care of mice and all procedures.

Isolation and Characterization of Brain MV

Mice were anesthetized and the heart was perfused with 20 mL of serum-free Dulbecco’s Modified Eagle’s Medium (DMEM) ( Gibco-Invitrogen, Grand Island, NY) to remove all blood components from the vasculature. After decapitation and careful removal of the meninges and large vessels, the cerebral cortex was dissected from the remainder of the brain. The cortices were triturated in serum-free DMEM with 4–5 strokes of a Dounce-type tissue grinder with a Teflon plunger. The disrupted tissue was allowed to settle into “pellet” and “supernate” fractions. The “pellet” fraction was passed through a 300 µm mesh nylon sieve. The material retained by the sieve (which consisted primarily of networks of MV) was collected by rinsing the sieve over a petri dish with jets of DMEM delivered from a pipette. The “supernate” fraction was passed through a 40 µm nylon mesh sieve. The filtrate, which contained mostly single cells, lipids, and cellular debris, was discarded. The material retained on the sieve consisted primarily of MV of shorter lengths, which were rinsed from the sieve and combined with the MV isolated from the 300 µm mesh sieve. The MV were washed once by a 5 min, 1,000g centrifugation through DMEM and then utilized for the MV experiments described herein. MV viability was confirmed after 48 h of culture using a ReadyProbes Cell Viability Imaging Kit (Molecular Probes/ThermoFisher Scientific, Waltham, MA).

Immunohistochemistry and immunofluorescence

Mice were euthanized and their brains removed and fixed in 10% neutral-buffered formalin. The fixed brains were paraffin-embedded and sectioned at 5 µm. Slide-mounted sections were de-paraffinized, blocked in phosphate buffered saline (PBS)/2% goat serum and exposed either to 2–5 µg/mL of biotinylated Solanum tuberosum lectin (Item B-1165, Vector Laboratories, Burlingame, CA) to label brain MV, or biotinylated HA binding protein (Item 385911, Calbiochem, San Diego, CA) to label brain-associated HA. Bound Solanum lectin was visualized with Alexa Fluor 488-streptavidin (Item S11223, Molecular Probes/ThermoFisher Scientific) and images were recorded using an epifluorescence microscope (Leica model DMR, Leica Microsystems, Wetzlar, Germany) equipped with a SPOT RT 1.4 mpixel color/monochrome CCD camera (Diagnostic Instruments, Sterling Heights, MI). Bound biotinylated HA binding protein was visualized with Vectastain Avidin-Biotin Complex (ABC) (Item PK-6105, Vector) in conjunction with 3,3′-diaminobenzidine (DAB) (Item SK-4105, Vector). The DAB-stained sections were viewed by conventional brightfield imaging using a Leica DM2500 microscope equipped with a SPOT Insight 4 mpixel color CCD camera (Diagnostic Instruments).

For immunofluorescence of MV, vascular tissues were placed on tissue culture plates, fixed for 1 h in 10% neutral-buffered formalin, blocked with PBS/2% goat serum and exposed to either to 2–5 µg/mL of biotinylated Solanum lectin (Vector Laboratories), 2–5 µg of a rabbit polyclonal antibody to smooth muscle actin (Item ab5694, Abcam, Cambridge, MA), or 2–5 µg of a polyclonal rabbit antibody to glial fibrillary acidic protein (GFAP) (Item ab7260, Abcam). Bound lectin or primary antibodies were visualized by exposure of the sections to (respectively) Alexa Fluor 488-streptavidin, or Alexa Fluor 594-goat anti-rabbit IgG (Items S11223, and A11012 all from Molecular Probes/ThermoFisher Scientific) and images were recorded using an ABI 7900 RT-PCR instrument with SYBR Green Master Mix (Bio-Rad) for mRNAs corresponding to murine HASes 1–3 and murine GAPDH. The following primer sets were used:

HAS1 F: AAGATGATCGTGAGTGCTCG
HAS1 R: GAGGAATTCGGAGGACC
HAS2 F: GCACCATGTTCTGCT
HAS2 R: CTCTGATGACGCGAATGC
HAS3 F: AGACTACAGGGGCAAACAG
HAS3 R: CGGAAAGTTTTAAGGGA
GAPDH F: CCACCCGAGGACTTGGAT
GAPDH R: GGTGCGAGGATGATGTCT

Image Analysis

For quantification of MV density in cerebral cortex sections stained with Solanum lectin or HA deposition in cerebral cortex sections stained with HABP/DAB, at least two digital images per section were obtained at 10x magnification. The images were opened in ImageJ (NIH image analysis freeware, http://imagej.nih.gov/ij/) and the brightness and contrast adjusted before converting to RGB-stacked images. Density of Solanum lectin-stained MV was expressed as an area fraction (area of the section stained with Solanum lectin/area of the standard field) × 100%. Deposition of HA in HABP/DAB-stained sections was also expressed as an area fraction, following modification of the threshold in the blue channel until the DAB-stained areas showed optimal contrast in the red channel.

Real-Time Polymerase Chain Reaction

Total cellular RNA was isolated from MV using TRIzol (Invitrogen/ThermoFisher Scientific). RNA purity and integrity was assessed by spectrophotometric analysis. A total of 1 µg of RNA was reverse-transcribed using an iScript kit (Bio-Rad Laboratories, Hercules, CA). Real-time polymerase chain reaction (RT-PCR) was performed using an ABI 7900 RT-PCR instrument with SYBR Green Master Mix (Bio-Rad) for mRNAs corresponding to murine HASes 1–3 and murine GAPDH. The following primer sets were used:

HAS1 F: AAGATGATCGTGAGTGCTCG
HAS1 R: GAGGAATTCGGAGGACC
HAS2 F: GCACCATGTTCTGCT
HAS2 R: CTCTGATGACGCGAATGC
HAS3 F: AGACTACAGGGGCAAACAG
HAS3 R: CGGAAAGTTTTAAGGGA
GAPDH F: CCACCCGAGGACTTGGAT
GAPDH R: GGTGCGAGGATGATGTCT
All experiments were performed in triplicate and normalized to GAPDH mRNA (23). Fluorescent signals were analyzed during each of 40 cycles consisting of denaturation (95°C, 15 s) and annealing (54°C, 15 s). Relative quantitation was calculated using the comparative threshold cycle method.

Quantification and Size Determination of HA in Cerebral Cortex Homogenates

Mouse cerebral cortex was homogenized, desiccated, and measured by a competitive enzyme-linked sorbent assay (ELSA) as previously described (24–26). Briefly, homogenates were dialyzed, digested with proteinase K (250 µg/mL) to degrade endogenous proteins and proteoglycans. After heat-inactivation of the proteinase K, the samples were assayed for HA. HA reactivity was quantitated according to a standard curve of purified HA.

To determine the size of HA, samples were first purified by anion exchange chromatography using DEAE-Sephacel (Sigma) mini column. The eluted fractions containing HA and glycoproteins were then applied to an analytical Sephacryl S-1000 column (GE Healthcare Life Sciences) to assess the size of HA. HA content in each fraction was assessed by HA-ELISA as described above.

Quantification of HA in Isolated Cortical MV

Equivalent portions of MV isolates from each mouse cerebral cortex were washed once with PBS and homogenized in PBS with a tissue homogenizer. An equal volume of Cell Lysis Buffer 2 (Item 895347, R&D Systems/Bio-Techne, Minneapolis, MN) was added and tissues were lysed at room temperature for 30 min with gentle agitation. Debris was then removed by centrifugation. An aliquot of the lysate was removed and assayed to determine HA levels using a quantitative sandwich enzyme immunoassay technique with a Hyaluronan Quantikine ELISA kit (R&D Systems) per the manufacturer’s instructions. HA content in the samples was normalized to the total protein content of each sample.

Quantification of HAS2 in Isolated Cortical MV

Equivalent portions of MV from each mouse cerebral cortex were washed by centrifugation through PBS and then triturated with a tissue homogenizer in PBS. Cells were lysed in the homogenates by freeze (−20°C)-thawing the samples three times. The lysates were then centrifuged for 5 min at 5,000g, the pellets discarded, and the supernates evaluated by bicinchoninic acid assay to determine protein concentrations. An aliquot of each sample was removed and assayed to determine HAS2 levels using a quantitative sandwich ELISA for mouse HAS2 (Item LS-F16174, LifeSpan BioSciences, Seattle, WA) per manufacturer’s instructions. HAS2 content in the samples was measured relative to the total protein content of the MV samples.

Quantification and Size Determination of HA Synthesized by Isolated Cortical MV In Vitro

HA synthesis by MV cultured in vitro was assessed as follows: freshly obtained MV from cerebral cortex were radiolabeled for 48 h with 50 µCi/mL of [3H]-glucosamine (Perkin Elmer, Waltham, MA) in DMEM with 5% fetal bovine serum. Supernates were isolated by centrifugation and digested with proteinase K (250 µg/mL) for 24 h at 60°C. Following digestion, proteinase K was inactivated by heating to 100°C for 20 min. HA and other glycosaminoglycans were separated from unincorporated [3H]-glucosamine by chromatography on Sephadex® G-50 columns (GE Healthcare, Wauwatosa, WI). Macromolecular fractions containing identical [3H] counts were incubated with or without 0.5 U/mL of Streptomyces HYAL (Sigma–Aldrich, St. Louis, MO) for 18 h at 37°C and analyzed by size exclusion chromatography on a 1.2 cm × 58 cm Sephacryl® S-1000 column (GE Healthcare). Fractions were eluted in 0.5 M sodium acetate/0.025% 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.0, and the radioactivity was measured by liquid scintillation counting. For each fraction, radioactivity associated with HA (defined as HYAL-sensitive glycosaminoglycan) was calculated by subtracting HYAL-resistant radioactivity from that of the undigested total.

Statistical Analysis

Initial comparisons for significant differences among aged, middle-aged, and young mice were made by ANOVA. Significant differences between aged and young mice were then determined using a paired Student’s t-test with unequal variance. Statistical significance was defined as p < .05.

Results

Changes in Microvascular Density and HA Accumulation in the Cerebral Cortex With Aging

The cerebral cortices of young, middle-aged, and aged mice were initially examined for microvascular density. As noted by others, we confirmed that the density of cerebral cortex microvasculature in aged mice was significantly lower than that of young mice (Figure 1A). The density of cerebral cortex microvasculature in middle-aged mice was similar to that of young mice (Figure 1A). We then measured the accumulation of HA in mice from different age groups. HA has been reported by others to be present in high levels in aged brain (18). We found by immunohistochemistry that aged mice had a significantly greater amount of HA in their cerebral cortex than did young, but not middle-aged, mice (Figure 1C). The HA was distributed throughout the cortex, but had the highest concentration in the perivascular areas (Figure 1C, inset). No differences with age were detected by immunohistochemistry for the presence of markers for apoptosis (caspase 3) or microglia (F4/80 or iba1) (data not shown). As expected, HA chain length (MW) in cerebral cortex had a broad range of MW with a midpoint of approximately 200 kDa. The size distribution of HA was similar between the young and aged mice (Figure 1D), as determined by non-quantitative sizing columns. Evaluation of brain cerebral cortex tissue for changes in mRNA transcripts for HA-associated proteins found no differences with age in neuron, brevican, aggregan, versican, bikunin, tenascin-R, tumor necrosis factor-stimulated gene 6 protein, HAS1, 2 or 3, or HYAL1, 2, or 3 by RT-PCR (data not shown).

HA and Mediators of HA Accumulation in Isolated Cortical MV with Aging

To focus specifically on the HA associated with brain microvasculature, we isolated MV (that were perfused to remove blood components) from the mouse cerebral cortex. As expected, these MV were comprised of endothelial cells (Figure 2A) with a network of supportive pericytes (Figure 2B) and associated astrocytes (Figure 2C). Analyses of HA content showed significantly higher levels of HA in MV from aged brains versus young brains (Figure 2D). We then sought a mechanism that might account for the increased levels of HA in aged MV by measuring important regulators of HA accumulation: (a) the HASes that mediate HA synthesis, (b) the HYALs...
that mediate HA degradation, and (c) HA-associated hyaladherins. Of the three HAS isoforms, we found that HAS2 mRNA was predominant in cortical MV (Figure 3A). Levels of HAS2 mRNA were significantly increased in cortical MV isolated from aged mice compared to corresponding MV from young and middle-aged mice (Figure 3A). Age-associated increases were not observed for HAS1 and HAS3 mRNA (Figure 3A). The age-associated increase in HAS2 mRNA corresponded to increased levels of HAS2 protein in cortical MV from aged versus young and middle-aged mice, as determined by quantitative ELISA (Figure 3B). With respect to the HYALs, mRNAs for HYALs 1–3 were examined, based on the potential for expression of these isoforms in brain parenchyma (10). We did not find age-related differences in mRNA levels for these HYALs (Supplementary Figure 1). Moreover, mRNAs corresponding to HA-associated hyaladherins that can inhibit HA degradation (neurocan, brevican, aggrecan, versican, bikunin, tenascin-R, and tumor necrosis factor-stimulated gene 6 protein) showed no significant age-related differences in expression (data not shown).

HA Synthesis and Size Distribution in Isolated Cortical MV With Aging

Given our findings that HAS2 mRNA and HAS2 protein were upregulated in MV isolated from aged versus young mice, we sought to determine whether the differences in HAS2 expression would translate into differences in de novo synthesis of HA by isolated MV placed in culture. Indeed, over 48 h in vitro, MV from aged mice synthesized significantly more HA than did MV from young mice (2.5-fold more), as shown by incorporation of [3H]-glucosamine (Figure 4A). Similar to extant HA, the newly synthesized HA from the two age groups exhibited similar MW profiles (Figure 4B). It should be noted, however, that greater HA synthesis resulted in higher levels of all MW forms of HA in the aged MV relative to young MV.

Figure 1. The density of microvessels (MV) in the mouse cerebral cortex decreases and hyaluronan (HA) content increases with age. (A) Histogram shows the density of MV, expressed as the percentage of Solanum lectin-stained area per standard field, as evaluated by ImageJ. The density of cortical MV in aged mice is significantly lower than that of young and middle-aged mice. Differences in young versus middle-aged samples were not statistically significant. ***p < .0005 aged samples versus young and middle-aged samples. (B) Typical appearance of Solanum-stained sections of cerebral cortex from young, middle-aged, and aged mice. (C) The content of HA in sections of cerebral cortex of aged mice was significantly higher than HA levels in the cerebral cortex of young, but not middle-aged mice, as determined by immunohistochemistry (deposition of HA in the sections is expressed as the percentage of HA binding protein/3,3′-diaminobenzidine (DAB)-stained area per standard field, as evaluated by ImageJ). *p < .05 aged versus young samples. n = 5 for each age group. (D) The molecular weight (MW) distribution of HA from the cerebral cortex of young and aged mice was similar, as determined by nonquantitative sizing columns. The peak of the MW profile of a purified 200 kDa HA standard (marked by the black bar) is shown for reference. The MW of HA increases to the left of the 200 kDa marker and decreases to the right of the marker. n = 5 for each age group.

Figure 2. Hyaluronan (HA) content in isolated cortical microvessels (MV) increases with age. (A-C) Representative fluorescence images of isolated cortical MV labeled to reveal endothelial cells (Solanum lectin, panel A), pericytes (anti-smooth muscle actin antibody, panel B), and astrocytes (anti-GFAP antibody, panel C). In A–C, cell nuclei are labeled with DAPI. (D) HA content of isolated brain MV increases with age, as determined by ELISA. *p < .05 aged versus young samples. n = 5 for young mice and n = 7 for aged mice.
HA is a major component of the ECM associated with MV and its synthesis generally increases in areas of injury (7,13,19,20,29). HA localization in brain ECM varies with the pathologic condition. For example, HA is the main component of white matter hyperintensities that can be seen by magnetic resonance imaging of regions of ischemia and demyelination (30). It has been proposed that in ischemic regions of the brain, HA inhibits oligodendrocyte precursor cell maturation and modulates signals that can potentiate additional neuronal injury (19,31,32). As in other organs, the effects of HA on cellular functions in the brain are influenced by HA MW. It is generally accepted that HA of low MW (e.g., <50 kDa) contributes to inflammatory processes in the brain by directly promoting cell influx, as well as inducing cells of the brain microvasculature to release cytokines. HA-associated impacts on neuroinflammation are implicated in stroke, trauma, autoimmune inflammation (e.g., multiple sclerosis), and infections (encephalitis). In animal models and human disease, HA mediates many of its central nervous system effects by interactions with the HA receptor, CD44, and the inflammatory receptors TLR2 and TLR4 (32–34).

The relationship between HA, injury, and inflammation underscores the potential connection between HA accumulation and MV loss with aging. Although in most tissues, newly synthesized HA has a relatively high MW (e.g., 200 kDa to more than 4,000 kDa) and has anti-inflammatory properties, HA is continually degraded into shorter chain lengths that can promote inflammation. Consequently, sites of increased HA synthesis in the brain may ultimately serve as generators of proinflammatory HA that might promote MV regression. Accumulation of HA around MV could also impact the function of the blood-brain barrier. It is generally accepted that the blood–brain barrier is more vulnerable to disruption with age, but existing studies suggest a range of mechanisms that do not focus on the ECM (2,4,5). Given the ubiquitous presence of HA in the cerebral microvasculature, we posit that changes in HA chain length or in the thickness of the HA layer in the abluminal or luminal glycocalyx of endothelial cells might contribute to this vulnerability.

The finding of increased levels of HAS2 as a mechanism accounting for greater quantities of HA in MV isolated from aged versus young cerebral cortex was somewhat surprising, as aging is generally associated with deficits in synthetic enzymes. For example, aged tissues generally show a reduction in biosynthesis of growth factors, hormones, and ECM (9,35). We found that the relative increase in HAS2 in MV isolated from aged versus young cortex was accompanied by a corresponding increase in synthesis of HA in vitro by the aged versus young MV. Although differences in low MW HA are difficult to detect, we did not find an
age-related difference in the MW profiles of HA in cerebral cortex or HA synthesized in vitro by the isolated cortical MV. However, it should be noted that the greater overall HA synthesis in aged versus young MV results in higher levels of HA throughout the size range, which includes low MW HA. Interestingly, although upregulation of HASEs in a variety of cell types can be accompanied by increases in HYALs, we did not see a corresponding increase (or decrease) in HYALs in aged versus young MV. We also found no age-related differences in the HA-binding hyaladherins that can inhibit HA degradation. These results contrast with the widely accepted concept of a senescence-associated secretory profile in aged ECM that predicts an increase in ECM degradative enzymes, such as HYALs (36,37). Indeed, even in the brain there are reports of both increases and decreases in proteolytic activity associated with brain aging and neurodegneration (38–42). These data confirm our premise that each organ, and also specific cellular environments with tissues (e.g., MV), develop unique ECM microenvironments with aging (43,44).

This study is limited in that it provides only the foundation for experiments that establish a direct association between higher amounts of HA and deficits in cortical MV with aging. The objective will be to utilize in vitro data to then determine if inhibiting HA accumulation will mitigate the changes in microvascular density or function in the aged brain in vivo.

Conclusion

In summary, we found that brain cortex and cortical MV from aged mice accumulate and synthesize significantly more HA than those of young mice. Although we did not identify an age-related increase in HA degradation within normal brains, or isolated brain MV, the greater accumulation of HA of all MW classes in aging brain MV might serve as a source of proinflammatory low MW forms of HA. We propose that increased HA synthesis in brain MV alters the neuroinflammatory environment and contributes to age-related alterations in brain microvascular density and function.

Supplementary Material

Supplementary data is available at The Journals of Gerontology, Series A: Biomedical Sciences and Medical Sciences online.

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Conflict of Interest

The authors have no conflicts to disclose.

References


