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Nanomechanical measurement of astrocyte stiffness correlated with cytoskeletal maturation

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Abstract: Astrocytes are known to serve as scaffolding cells that shape the brain. The physical properties of astrocytes, such as stiffness, are important for their scaffolding function. These properties may be altered in certain pathological conditions, such as in brain cancer. However, actual stiffness of astrocytes is not yet well understood. Here, we report that the astrocyte stiffness is positively correlated with the density of cytoskeletal proteins, such as actin filaments, microtubules, and intermediate filaments. The value of the stiffness of astrocytes as measured by atomic force microscopy (AFM) increases 38-fold in five-week-old rats compared to postnatal-day zero pups. Using multicolor confocal microscopy, we found that the complexity of cytoskeletal proteins, such as actin filaments, microtubules, and intermediate filaments, increase as the animal gets older. Our findings indicate that the change of stiffness positively correlates with the maturation of cytoskeletal proteins, and suggest that AFM can be useful as an analytical and diagnostic tool for neuroscience. J Biomed Mater Res Part A: 00A:000–000, 2014.

Key Words: atomic force microscopy, astrocyte, cell stiffness, *ex vivo* culture, cytoskeletal structure

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INTRODUCTION

A subtype of glial cells in the central nervous system (CNS), astrocytes occupy more than half of the total cellular population in the adult mammalian brain. They provide structural, metabolic, and trophic support for neurons and surrounding endothelial cells.[1] Recently, the known functions of astrocytes have been expanded to include synaptic modulation.[2] Astrocytes in the supraoptic nucleus of lactating mouse brains retract their processes leading to reduction in memory.[3] This suggests that the astrocyte's structure is important for modulating the synapse.[4] Thus, the physical property of the astrocyte may be a clue to understanding synaptic modulation.

Physical changes in a cell are highly correlated to the remodeling of its intracellular components.[5] Among them, cytoskeletal structures, a supramolecular network comprised of actin filaments, intermediate filaments, and microtubules, assume the most important role in maintaining the cell shape, morphology, signaling, and intracellular transport.[6,7] Cytoskeletal structures are dynamically reconstructed during cell migration, adhesion, proliferation, and differentiation. Actin filaments, which are double-stranded, thin, and flexible structures (5–9 nm), give support and structure to the plasma membrane and are found close to the cell membrane. Microtubules, long, rigid, and cylindrical structures (~25 nm), are organized around the centrosome, and extend through the cytoplasm from organelles. Intermediate filaments of ~10 nm diameter have fibrous and rope-like structures, and are found either as a network structure surrounding the nucleus or organized through cytoplasm.[8,9]

Atomic force microscopy (AFM) has been established as a versatile tool for probing biological samples such as cells,

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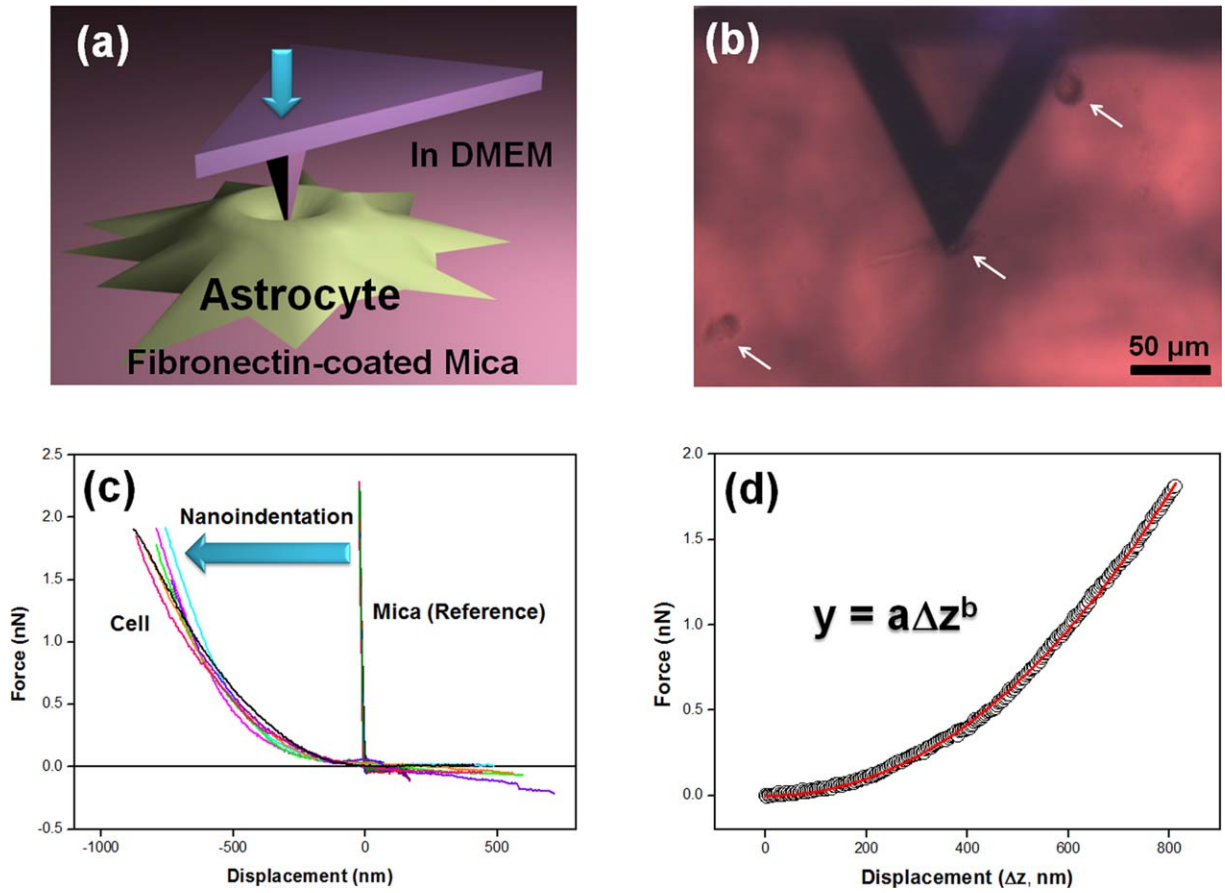

FIGURE 1. (a) View of AFM nanoindentation on astrocytes *in vivo* cultured on fibronetin-coated mica (left) and its illustration (right), (b) representative force vs. distance (*F-D*) curves achieved from indentations against astrocytes, and mica surfaces as a reference. (c) Fitting curve and its equation (inset) for the plot converted from *F-D* curve. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

viruses, and various biomolecules.[10,11] In terms of the cellular system, AFM provides detailed knowledge of intrinsic biophysical properties such as stiffness and viscoelasticity, as well as topographical information of cellular architectures. In particular, biophysical changes related to the remodeling of the intracellular cytoskeleton have been investigated in various viewpoints.[12] The application of AFM has expanded to biomedical fields as a nanomechanical transducer. AFM also allows biophysical analysis to more effectively evaluate clinical issues such as disease. Kol et al. reported the maturation-induced "stiffness switch" in HIV by measuring the stiffness of HIV particles using AFM nanoindentation, which establishes the groundwork for mechanistic studies of how retroviral particles can regulate their mechanical properties in order to affect biological functions.[13] By using AFM measurement, Lieber et al. investigated how age-related changes in the mechanical properties of cardiac myocytes contribute to the global changes in left ventricular (LV) diastolic function.[14] In terms of disease diagnosis, Cross et al. used AFM to examine the nanomechanical responses of metastatic cancer cells and benign mesothelial cells taken from human body cavity fluids. They found that metastatic cancer cells are more than 70% softer than the benign cells lining the body cavity.[15]

It is well known that astrocytes growth is critical for the repair and growth of neural cells.[16] Therefore, understanding the age-dependent mechanical properties of astrocytes will be helpful in predicting *in vivo* behavior of neural cells, as well as with investigating neural repair in clinical settings.[17,18] Here, we report the relationship between age-dependent nanomechanical changes and the cytoskeletal network of astrocytes, based on immunofluorescence images of astrocytes as well as the changes in stiffness.

EXPERIMENTAL SECTION

Preparation of fibronectin-coated mica

A coating solution containing 0.001% fibronectin and 0.02% gelatin was prepared using autoclaved distilled water. Mica chips (10 mm) were exposed to O_2 plasma for 1 min and then, incubated in the coating solution at 37°C overnight. The fibronectin-coated mica chips were dried and stored at 4°C until cell attachment.

Astrocyte culture on mica surface

Cerebral cortices from neonatal Sprague–Dawley rat brains (P0 to week 8) were removed and carefully dissected in accordance with the animal care and use regulations of the Korea Institute of Science and Technology (KIST). The tissue

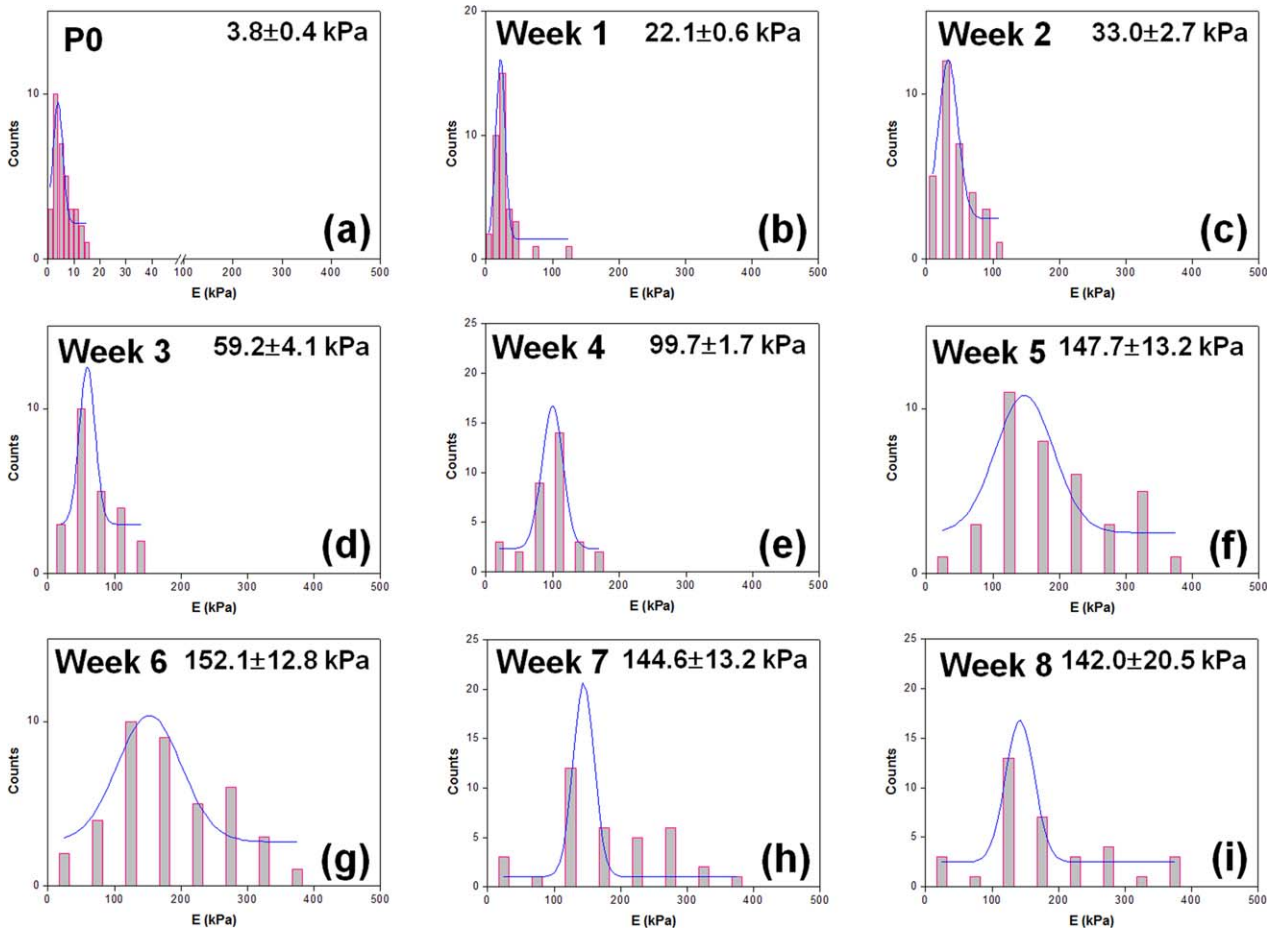

FIGURE 2. AFM-based stiffness histogram of astrocytes obtained from (a) 1 day old, (b) 1 week old, (c) 2 weeks old, (d) 3 weeks old, (e) 4 weeks old, (f) 5 weeks old, (g) 6 weeks old, (h) 7 weeks old, and (i) 8 weeks old rats. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was dissociated in Dulbecco's modified eagle's medium (DMEM) containing 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA), and passed through a 70-mm pore nylon mesh. After centrifugation, the cell pellet was resuspended in DMEM medium (GIBCO, USA), supplemented with 10% fetal bovine serum (Cambrex, USA) and 2% penicillin/streptomycin (Cambrex, USA). The cells were then plated onto fibronectin-coated mica at a density of 2×10^4 cells/mL. The medium was renewed every 2–3 days. After 5 days in culture (2 days for P0), the non-adherent microglia cells were collected, and purified by preferential adhesion. These procedures resulted in over 98% pure astrocyte cell populations.

AFM measurement

All force spectroscopy measurements were obtained with an AFM model XEI-120 (Park Systems, South Korea). A 100-μm scanner and Teflon open liquid cell were used for the experiments. Veeco Si_3N_4 cantilevers (model NP-10) with nominal spring constant of 0.12 N/m, tip radius of ~20 nm and tips with 20 degrees half curvature were used for the measurements. The astrocytes-attached mica surfaces were glued onto steel discs, and mounted on open liquid cells. The liquid cells were then filled with DMEM for force versus distance (*F-D*) curve measurements (the buffer was injected very carefully to avoid bubble creation, and prevent liquid from over-flowing in the liquid cell). After fine adjustment

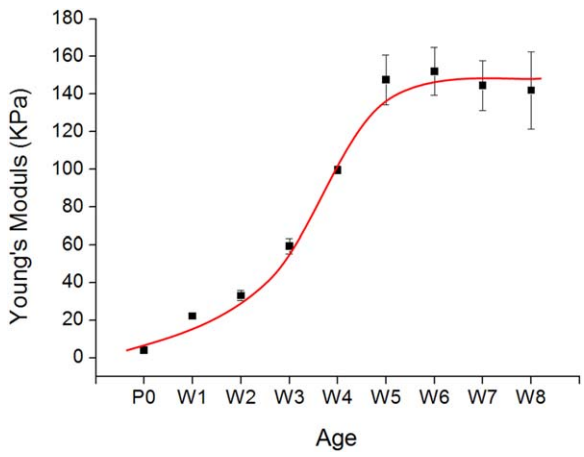

FIGURE 3. Plot of Young's modulus of astrocyte as a function of rat age and its fitting curve. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

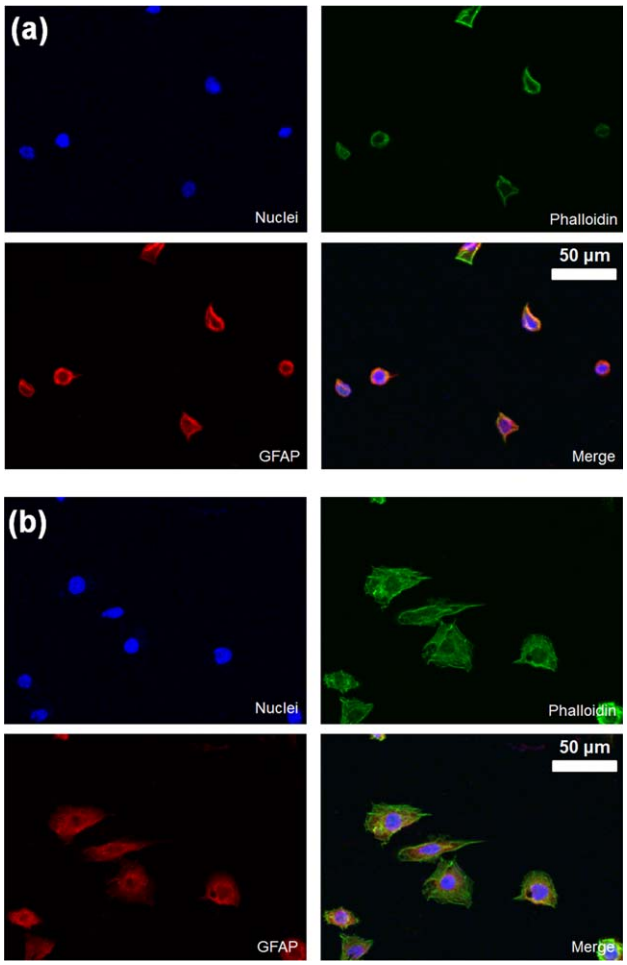

FIGURE 4. Representative immunofluorescence images of nuclei, actin filaments, and GFAP (glial fibrillary acidic protein); (a) P0 astrocytes and (b) 5-week-old astrocytes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of the laser spot on the apex of the cantilever, the cells were located by the camera connected to the AFM system, and the tips were brought to the center of the cells. Then, 100 *F-D* curves were performed on the area at room temperature with a tip velocity of 300 nm/s (total 1024 points). For each sample (different ages or types), about 60 cells were selected for measurement. Other parameters were optimized for each experiment.

Immunofluorescence imaging

Cultured cells on the mica surface were confirmed as astrocytes by immunofluorescence. The cultured cells were fixed by immersing the mica disks which were mounted onto in 4% paraformaldehyde/0.1M phosphate buffered saline (PBS) buffer for 20 min at room temperature. Thereafter, they were transferred to a 0.1M PBS buffer containing 0.1% Triton X-100 (Sigma-Aldrich, USA), and incubated for 20 min at 4°C. Glial fibrillary acidic protein (GFAP) polyclonal antibody was then applied as primary antibody at a dilution of 1:200 (Millipore, USA) before overnight incubation at 4°C. The next day, the sections were washed three times in PBS and incubated in a secondary antibody solution (Cy5 donkey anti-chicken IgY at the dilution of 1:100; Millipore, USA). Coverslips were mounted by VECTASHIELD mounting media containing DAPI (Vector Labs, USA).

The cytoskeletal structures of astrocytes were also visualized by immunofluorescence staining. Astrocytes on the mica chips fixed in 4% paraformaldehyde and blocked by 0.1% Triton X-100 were incubated overnight at 4°C in a solution containing FITC-Phalloidin (Sigma-Aldrich), β-tubulin monoclonal antibody (Cell Signaling Technology, USA), and Vimentin monoclonal antibody (Abcam, USA) at a dilution of 1:1000. Phalloidin, β-tubulin, and Vimentin represent actin, microtubule, and intermediate filaments, respectively. After rinsing with PBS three times, the astrocytes were incubated in goat polyclonal antibody solution containing Cy5 anti-mouse antibody (Abcam, USA) and chicken Cy3.5 anti-mouse antibody (Abcam, USA) at a dilution of 1:200 for 1 h at room temperature. Images of the staining distribution were obtained using a confocal laser microscope (FV1000; Olympus, Japan).

RESULTS AND DISCUSSIONS

Several techniques have been used for evaluating the biophysical properties of cells and other biological samples. Among them, AFM gained popularity because it can be used to obtain a diverse range of biophysical information such as cell stiffness and molecular interaction as well as cell topography with nanoscale resolution. The cytospin and *ex vivo* culture methods are two techniques used to measure cell stiffness and elasticity.[15] The advantage of using the former is that cell stiffness can be measured while avoiding artifacts that may arise from *ex vivo* culture. However, this method should be confined to floating cells cultured from a cell line, or taken from body cavity fluid. The cytospin method does not seem applicable to our approach because it is hard to discriminate astrocytes from debris including neurons and other glial cells in the brain-digested mixture. In this respect, we *ex vivo* cultured astrocytes separated from nine different aged rats on fibronectin-coated mica surfaces. The rats ranged from a 1-day-old pup to an 8-week-old adult rat. The pup astrocytes (P0) were incubated on fibronectin-coated mica for only 2 days because they proliferated so fast whereas astrocytes from 1-week-old to 8-week-old rats were incubated for 5 days. The stiffness of live astrocytes was measured by AFM in cell culture media [Fig. 2(a)]. Nanoindentation was undertaken at the central region (possibly the thickest region) of the astrocytes to minimize the substrate effect. The indentation curves measured on the astrocytes were compared with the *F-D* curve without deformation of the mica surface as a reference curve. Furthermore, since the rupture force between tip and cell is significant enough to have an effect on the slope of the *F-D* curve, we selected only the approach curve for analysis of cell indentation. Representative approach curves on mica surfaces and on astrocytes are shown [Fig. 2(b)]. The difference between the slopes of the *F-D* curves measured on hard surface and soft sample shows the deformation of the soft sample.

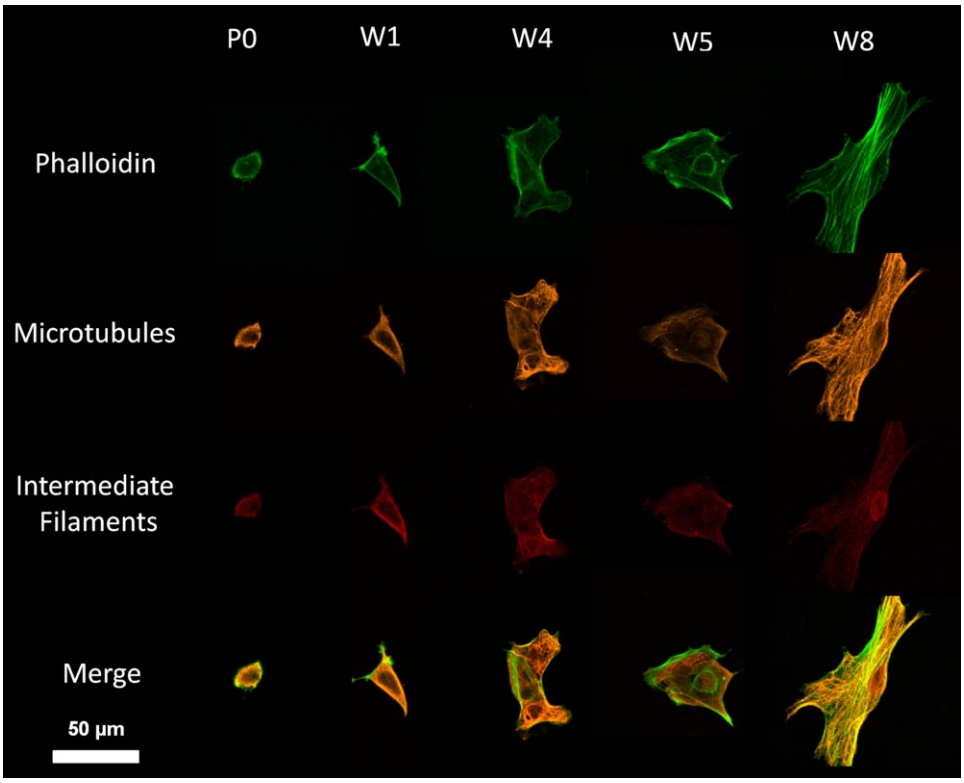

FIGURE 5. Immunofluorescence images of actin filaments, microtubules, and intermediate filaments of rat cortical astrocytes according to the indicated ages. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

To determine the Young's modulus of the astrocytes, we applied the Hertz–Sneddon model in which the force (F) versus indentation (Δz) curves are fitted with the equation $F = a{\cdot}\Delta z^b$ [Fig. 2(b)]. The constant a and the power parameter b represent the Young's modulus and the tip shape, respectively. For the four-sided pyramidal tip, the parameter b is about 2, and the corresponding equation to calculate the force will be:

$$F_{\text{pyramid}} = E^* \tan\alpha / 2^{1/2} \Delta z$$

where, α is the face angle of the four-sided pyramidal AFM tip, E^* is the relative Young's modulus:

$$1/E^* = \left(1-\mu^2{}_{\text{astrocyte}}\right)/E_{\text{astrocyte}} + \left(1-\mu^2{}_{\text{astrocyte}}\right)/E_{\text{tip}}$$

where, $\mu_{\text{astrocyte}}$ and μ_{tip} are the Poisson's ratios (from 0 to 0.5), and $E_{\text{astrocyte}}$ and E_{tip} are Young's moduli of the astrocyte cell and the tip, respectively.[2] The tip elastic (E_{tip}) is ~150 GPa which is around 10^5 times greater than E_{sample}; therefore the simplified equation for cell elasticity can be, $1/E^* = (1 - \mu^2_{\text{astrocyte}})/E_{\text{astrocyte}}$. The Young's modulus of the astrocyte is then $E_{\text{astrocyte}} = 2^{1/2}\ (1 - \mu^2_{\text{astrocyte}})/\tan\alpha\ a$. The parameter a can be acquired from fitting the *F-D* curve [Fig. 2(c)]. In order to obtain the most probable value of the Young's modulus, 60 astrocytes were measured at each age and the data were used for a Gaussian fit.

Our ultimate goal for this study was to understand how elastic the membranes of astrocytes are. To determine whether the elasticity of *ex vivo* cultured astrocytes decrease with increasing age, we measured the stiffness using AFM (Fig. 3). The stiffness (Young's modulus) histograms of astrocytes separated from rats ranging a day old pup to an 8-week old adult are shown in Figure 3. The stiffness values were estimated by Gaussian fitting, and their correlation is presented as a function of the age (Fig. 4). The astrocyte stiffness increased until week 5 from 3.8 ± 0.4 kPa to 147.7 ± 13.2 kPa (~39 times), and thereafter, the astrocytes maintained their stiffness without significant change up to week 8 (~145 kPa). A breakthrough point was observed between weeks 3 and 4, suggesting that the rat brain could be matured suddenly around 3 or 4 weeks, and brain tissue growth was practically terminated around week 5. Interestingly, increasing deviation of stiffness from week 5 to week 8 also shows a growing trend as a function of the age. We assumed that this was the anisotropic and inhomogeneous cell morphology induced by size growth of a single cell, which would be supported by cytoskeletal imaging.

To clarify whether the stiffness data acquired by AFM were recorded from astrocytes or not, the cultures were immunocytochemically stained with antibodies for both GFAP [green color in Fig. 4(a,b)] and intermediate filaments in order to observe the complexity of the cell inside. We observed interwoven structures of red-colored intermediate

filaments [red color in Fig. 4(a,b)] in the cytoplasm of astrocytes for all ages, confirming that most of the cells attached on the mica were indeed astrocyte cells [Fig. 4(a,b), merged]. For this study, GFAP was used as a specific maker protein for the intermediate filament of astrocytes. The intensity of the red dye indicating intermediate filaments in 5-week-old astrocytes obviously shows the increase in complexity [red color in Fig. 4(b)] compared to that of P0 astrocytes [red color in Fig. 4(a)].

Cell stiffness is known to be determined by cell membrane, subcellular structure including cytoskeletal structure, and so forth, as well as cell geometry such as size, shape, and thickness. It does not seem that one factor solely influences cellular biomechanics. We next investigated whether the age-dependent change of astrocyte stiffness is related to the density of cytoskeletal proteins. Immunocytochemistry was used to examine the density of cytoskeletal proteins. The cytoskeletal proteins such as actin filaments, intermediate filaments, and microtubules were not fully developed in younger rats, but were well developed in rats aged 4, 5, and 8 weeks (Fig. 5). Astrocytic development is known to be greatly ramified after 4 weeks.[17] Immunocytochemical intensities representing actin, microtubules, and intermediate filaments significantly increased between weeks 4 and 8, confirming that our immunocytochemistry data from *ex vivo* astrocytes are quite similar to that of *in vivo* astrocytes. Our immunocytochemistry data indicate that the maturations of cytoskeletal proteins depend on age. Taken together with AFM and immunocytochemistry analysis, there is a correlation between maturation of cytoskeleton and the values measured by AFM. We hope that our findings about the age-dependent mechanical properties of astrocytes will be helpful in predicting *in vivo* behavior of neural cells, as well as with investigating neural repair in clinical settings

CONCLUSIONS

In conclusion, we report age-dependent changes in rat astrocytic stiffness, which are measured by AFM. Using multicolor confocal microscopy, the age-dependent nanomechanical changes in astrocytes were correlated with the maturation of intracellular structures, in particular, cytoskeletal elements such as actin filaments, microtubules, and intermediate filaments. Astrocytes, isolated from nine different aged rats ranging from 1-day-old pup to 8-week-old rat, were *ex vivo* cultured for 5 days and fixed onto fibronectin-coated mica surface. Individual astrocytes were evaluated with AFM, and the force–distance curves for over 60 astrocytes were recorded for each age. We observed that the stiffness of astrocytes increased from $\sim$4 kPa to $\sim$146 kPa as a function of age and the breakthrough occurred between week 3 and 4. Immunofluorescence images of astrocytes also showed excellent correlation with these nanomechanical results. Our findings indicate that the maturation of intracellular components including cytoskeletal structures determine the nanomechanical properties of astrocytes. We conclude that understanding the age-dependant mechanical properties of astrocytes is helpful to predict the *in vivo* function of these cells.

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REFERENCES

1. Nedergaard M, Ransom B, Goldman SA. New roles for astrocytes: redefining the functional architecture of the brain. Trends Neurosci 2003;26:523–530.
2. Haydon PG, Carmignoto G. Astrocyte control of synaptic transmission and neurovascular coupling. Physiol Rev 2006;86:1009–1031.
3. Oliet SH, Piet R, Poulain DA. Control of glutamate clearance and synaptic efficacy by glial coverage of neurons. Science 2001;292:923–926.
4. Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA, Oliet SH. Glia-derived D-serine controls NMDA receptor activity and synaptic memory. Cell 2006;125:775–784.
5. Hu W, Onuma T, Birukawa N, Abe M, Ito E, Chen Z, Urano A. Change of morphology and cytoskeletal protein gene expression during dibutyryl cAMP-induced differentiation in C6 glioma cells. Cell Mol Neurobiol 2008;28:519–528.
6. Schliwa M, van Blerkom J. Structural interaction of cytoskeletal components. Structural interaction of cytoskeletal components. J Cell Biol 1981;90:222–235.
7. Fletcher DA, Mullins RD. Cell mechanics and the cytoskeleton. Nature 2010;463:485–492.
8. Fuchs E, Cleveland DW. A structural scaffolding of intermediate filaments in health and disease. Science 1998;279:514–519.
9. Wang N, Stamenovic D. Contribution of intermediate filaments to cell stiffness, stiffening, and growth. Am J Physiol Cell Physiol 2000;279:C188–C194.
10. Karduna AR, Halperin HR, Yin FC. Experimental and numerical analyses of indentation in finite-sized isotropic and anisotropic rubber-like materials. Ann Biomed Eng 1997;25:1009–1016.
11. Sen S, Subramanian S, Discher DE. Indentation and adhesive probing of a cell membrane with AFM: theoretical model and experiments. Biophys J 2005;89:3203–3213.
12. Kuznetsova TG, Starodubtseva MN, Yegorenkov NI, Chizhik SA, Zhdanov RI. Atomic force microscopy probing of cell elasticity. Micron 2007;38:824–833.
13. Kol N, Shi Y, Tsvitov M, Barlam D, Shneck RZ, Kay MS, Rousso I. A stiffness switch in human immunodeficiency virus. Biophys J 2007;92:1777–1783.
14. Lieber SC, Aubry N, Pain J, Diaz G, Kim SJ, Vatner SF. Aging increases stiffness of cardiac myocytes measured by atomic force microscopy nanoindentation. Am J Physiol Heart Circ Physiol 2004;287:H645–H651.
15. Cross SE, Jin YS, Tondre J, Wong R, Rao J, Gimzewski JK. AFM-based analysis of human metastatic cancer cells. Nanotechnology 2008;19:384003.
16. Song H, Stevens CF, Gage FH. Astroglia induce neurogenesis from adult neural stem cells. Nature 2002;417:39–44.
17. Bushong EA, Martone ME, Ellisman MH. Maturation of astrocyte morphology and the establishment of astrocyte domains during postnatal hippocampal development. Int J Dev Neurosci 2004;22:73–86.
18. Lu YB, Franze K, Seifert G, Steinhauser C, Kirchhoff F, Wolburg H, Guck J, Janmey P, Wei EQ, Kas J, Reichenbach A. Viscoelastic properties of individual glial cells and neurons in the CNS. Proc Natl Acad Sci USA 2006;103:17759–1764.