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Fractone aging in the subventricular zone of the lateral ventricle

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ABSTRACT

In adulthood, the subventricular zone (SVZ) is one of the restricted places where neurogenesis persists. In this neurogenic niche, specialized extracellular matrix (ECM) structures termed fractones contact neural stem cells and their immediate progeny. Fractones are composed of ubiquitous ECM components including heparan sulfate proteoglycans such as perlecan and agrin. We have previously shown that fractones can capture growth factors and promote growth factor activity through a heparin binding mechanism in order to regulate neurogenesis. With aging, neurogenesis is known to decrease. However, the effect of aging on fractones structure and composition remains unknown. Here, we report that, while fractone number decreased, fractone size dramatically increased with aging. Despite the changes in fractones morphology, niche cells expressing glial fibrillary acidic protein kept direct contact with fractones. Furthermore, we have observed that heparan sulfate chains contained in fractones were modified with aging. However, FGF-2 was still captured by fractones via heparan sulfates. Together, our results suggest that the changes observed in fractones structure and composition are critically related to aging of the SVZ neurogenic niche.

1. Introduction

In the young adult mammalian brain, neurogenesis primarily occurs in the subventricular zone (SVZ), in the hippocampus and rostral migratory stream. In the SVZ, neural stem cells (NSC) have been identified as a slow dividing subset of glial fibrillary acidic protein (GFAP)-expressing cells (Doetsch et al., 1999). The maintenance of these NSCs has been shown to be dependent on their contact with the ventricle on their apical side and the underlying capillaries on their basal side. (Shen et al., 2008; Tavazoie et al., 2008) NSCs slowly divide to give rise to transit amplifying cells that in turns give rise to neuroblasts. These neuroblasts migrate to the olfactory bulb were they mature into interneurons (Lois and Alvarez-Buylla, 1994; Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002). However, neurogenesis is known to deteriorate in the aged mice (Maslov, 2004). Cell proliferation in the SVZ decreases with aging (Jin et al., 2003; Tropepe et al., 1997); accordingly, aged mice display a dramatic decrease in the number of NSCs (Maslov et al., 2004; Shook et al., 2012). Although a decreased rate of neurogenesis and changes in cellular organization is well documented, the mechanisms underlying the deterioration of the SVZ are still poorly understood. Furthermore, possible changes that may occur in the niche during aging remain to be clarified.

We previously characterized an ECM structure termed fractones that play a central role in the adult NSC niche. Fractones appear as a punctate structure by confocal microscopy but display a fractal structure by transmission electron microscopy (Mercier et al., 2002, 2003). This complex ultrastructure sets fractones apart from basement membranes. Moreover, fractones are found in the brain parenchyma while basement membranes in the brain locate along the vascular wall or at the interface between the meninges and the parenchyma. Fractones consist of the ubiquitous ECM components laminin, collagen IV, nidogen, and heparan sulfate proteoglycans (HSPGs) such as perlecan and agrin (Kerever et al., 2007, 2014; Mercier and Arikawa-Hirasawa, 2012; Mercier et al., 2002, 2003). HSPGs are complex molecules that play various biological roles through their protein core and their heparan sulfate (HS) chains (Kirn-Safran et al., 2009). HS chains consist of a repeated disaccharide motif of glucuronic acid and *N*-acetyl-glucosamine that are polymerized by exostosin1 (EXT1) and exostosin2 (EXT2).

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This polysaccharide is then modified in the Golgi apparatus by a series of enzymes, namely, *N*-deacetylase/*N*-sulfotransferases (NDST), C5 epimerase (GLCE), HS 2-*O*-sulfotransferase (HS2ST), HS 6-*O*-sulfotransferases (HS6ST), and HS 3-*O*-sulfotransferases (HS3ST) (Esko and Lindahl, 2001; Sugahara and Kitagawa, 2002). However, this series of reactions is not always performed to completion, leading to great variability in sulfation patterns. This sulfation heterogeneity plays an important role in the interaction specificity of HS with growth factors and their receptors (Ashikari-Hada et al., 2004; Maccarana et al., 1993; Powell, 2002; Pye et al., 1998; Sugaya et al., 2008).

We have previously shown that HS chains contained in fractones differ from those present in blood vessel basement membranes (Kerever et al., 2007). Furthermore, we have previously demonstrated that fractones can specifically sequester the growth factors FGF-2, BMP4 and BMP-7 via a heparin binding mechanism to modulate cell proliferation in the SVZ neurogenic niche (Douet et al., 2012, 2013; Kerever et al., 2007; Mercier and Arikawa-Hirasawa, 2012; Mercier and Douet, 2014a,b). Although our previous studies demonstrate a critical role of fractones in adult neurogenesis, the effect of aging on fractones structure and composition remains to be elucidated.

Here, we will examine the effect of aging on the ECM contents of the SVZ niche. We will describe changes in fractone morphology and composition as well as their relationship with the niche cells. Our results show that fractone size drastically increases while fractone numbers decrease in the SVZ of the aged mouse. We also show that SVZ astrocytes keep contacts with fractones. However, the morphology of the GFAP$^+$ cells is modified with aging. Immunoreactivity for specific HS epitopes is decreased in aged fractones. In old mice, fractones bind FGF-2 via heparan sulfates. Together, our results suggest that decrease capacity of the aging SVZ neurogenic niche is associated with fractone-morphology and heparan sulfate content.

2. Materials and methods

2.1. Animals

In this study, young (10–12 weeks) and aged (98–100 weeks) mice were used. All animal protocols were approved by the Animal Care and Use Committee of Juntendo University.

2.2. Histology

After mice were deeply anesthetized, brains were dissected and immersed in −40 °C isopentane. 25 μm-thick sections were obtained using a Leica cryostat. Sections were stored at −30 °C until immunostaining.

2.3. Heparitinase-1 treatment

Prior to fixation in 4% paraformaldehyde (PFA), frozen sections were incubated in heparitinase buffer (50 mM Tris–HCl; 10 mM $CaCl_2$; 3 mM NaCl; pH = 7) in the presence or absence of heparitinase-1 (EC4.2.2.8 from Sigma; 5 mU/ml) for 2 h at 37 °C.

2.4. Immunohistochemistry

Sections were post-fixed in cold PFA for 10 min and washed in PBS. Sections were then placed in a 0.5% Triton X-100/PBS solution for 15 min, followed by 15 min of a blocking solution (0.2% gelatin/PBS). Primary antibodies were applied for either 2 h at room temperature or overnight at 4 °C in blocking solution. The following primary antibody dilutions were used: rat anti-perlecan (1:400, clone A7L6, Chemicon, Temecula, CA), rabbit polyclonal anti-laminin (1:1000, L9393, Sigma, St Louis, MO), rabbit polyclonal anti-agrin (1:1000, kind gift of Dr. Sasaki), mouse anti-heparan sulfate (*n*-sulfated glucosamine, 10E4 epitope, 1:400, Seikagaku Corporation, Tokyo, Japan), mouse anti-heparan sulfate (*n*-unsubstituted glucosamine, JM403 epitope, 1:400, Seikagaku Corporation, Tokyo, Japan), mouse anti-delta heparan sulfate (3G10 epitope, 1:400, Seikagaku Corporation, Tokyo, Japan), rabbit anti-collagen IV (ab6586; 1:400, Abcam, Cambridge, MA, rabbit anti-GFAP (1:400, Dako, Glostrup, Denmark), mouse anti-GFAP conjugated to Alexa Fluor 647 (1:400, Cell Signaling Tech, Boston, MA). To reveal FGF-2 binding, recombinant human FGF-2 protein (Peprotech, Rocky Hill, NJ, USA) was linked to fluorescent tag using Dylight-650 labeling kit from Pierce (Thermo scientific, Rockford, Il, USA). FGF-2-650 was then use together with primary antibody (2.5 μg/ml). Sections were then rinsed, and secondary antibodies were applied for 40 min at room temperature. The following fluorochrome-conjugated secondary antibody dilutions were used: donkey anti-mouse-CY5, donkey anti-goat-FITC (1:400, Jackson ImmunoResearch Laboratories, West Grove, USA), goat anti-rabbit Alexa Fluor 488, and goat anti-mouse-FITC (1:400 Molecular Probes, Invitrogen Corporation, Carlsbad, USA). Sections were rinsed in PBS before being treated in 2 N HCl for 30 min at 37 °C and then incubated for 10 min in a 0.1 M borate buffer (pH 8.5). Sections were rinsed before overnight incubation in rat anti-BrdU (1:800, AbD Serotec, MorphoSys AG, Planegg, Germany). Sections were rinsed and then incubated for 1 h with donkey anti-rat-CY3 (1:400, Jackson, ImmunoResearch Laboratories, West Grove, USA). Sections were rinsed and then incubated for 10 min in bis-benzimide (1:3000, Molecular Probes, Invitrogen Corporation, Carlsbad, CA). After extensive washing, sections were mounted in fluoro-gel with tris buffer (Electron Microscopy Sciences, Hatfield, USA).

2.5. Whole-mount immunostaining

The SVZ (the caudate putamen side only) was dissected as previously described (Mirzadeh et al., 2008). Briefly, after brain extraction a coronal cut was made at bregma-1 mm. Then after separating the two hemispheres with a sagittal cut at the midline, the wall facing the midline was carefully removed. The SVZ was further dissected by cutting above the fornix and below the corpus callosum. Finally, we isolated the SVZ from the caudate putamen by carefully cutting a thin layer of the ventricle wall (<500 μm). Explants were incubated in PFA overnight at 4 °C and then clarified as previously described (Susaki et al., 2014). Briefly, whole-mounts were incubated for 2 days at 37 °C on a moving plate in a solution composed of 5 wt% urea (Nacalai Tesque Inc., 35904-45, Japan), 25 wt% *N,N,N′,N′*-tetrakis (2-hydroxypropyl) ethylenediamine (Tokyo Chemical Industry CO., LTD., T0781, Japan), and 15 wt% Triton X-100 (Nacalai Tesque Inc., 25987-85, Japan). Whole-mounts were washed in PBS Triton-X100 (0.1%) overnight and then incubated with 10E4 and GFAP antibodies as described above for three days at 37 °C on a moving plate. Whole-mounts were washed in PBS Triton-X100 (0.1%) overnight and then incubated with secondary antibodies for three days at 37 °C on a moving plate. Next, whole-mounts were incubated in a solution composed of 50 wt% sucrose (Nacalai Tesque Inc., 30403-55, Japan), 25 wt% urea, 10 wt% 2,20,20′-nitrilotriethanol (Wako Pure Chemical Industries Ltd., 145-05605, Japan), and 0.1% (v/v) Triton X-100. Image acquisition was performed with a Carl Zeiss LSM 780 confocal microscope with 63X Plan-Apochromat (N.A.: 1.2). Image processing was performed using Imaris (Bitplane software).

2.6. Quantitative real time PCR

The SVZ was dissected as described above. RNA from both SVZ (caudate putamen side) was extracted using TRIzol reagent (Invitrogen). cDNA was synthetized using the RT^2 First stand kit from Qiagen. Real time PCR was performed using RT^2 qPCR primer assays and RT^2 SYBR green mastermix from Qiagen on a Fast 7500 Real Time Cycler (Applied Biosystems). Beta-actin was used as endogenous control. Primers from Qiagen were as follows, *Ext1*, *Ext2*, *Ndst1*, *Ndst2*, *Glce*, *Hs2st1*, *Hs6st1*, *Hs6st2*, *Hs3st4*.

2.7. Quantification and statistical analyses

Analysis was performed using a Zeiss LSM780 microscope. Confocal images represent the maximum intensity projection of a stack of 11 images taken with a step size of 10 μm in the *z* axis. Fractones and blood vessels are discriminated by their three-dimensional morphology. Fractones never present the characteristic lumen of blood vessels. For fractones size, fractones on the open portion of the lateral ventricle (caudate putamen side only) were measured (young: $n = 4$; aged: $n = 9$; the size of more than 100 fractones per animal was assessed). Fractones number was determined by counting the number of fractones in one field (20×) on the open portion of the lateral ventricle on the caudate putamen side ($n = 3$; three field counted per animal). Immunofluorescence intensity within fractones ($n = 3$; three field counted per animal) was measured using ImageJ (ImageJ 1.45 o, Wayne Rasband, NIH, USA) on 8-bit tiff image taken with a 20× objective. Statistical data are presented as mean ± SEM and analyzed using the Unpaired Student's *t*-test with confidence intervals of 99% (Graph-Pad Prism version 5.0 for Mac OS X, Graph-Pad Software, San Diego, CA).

3. Results

3.1. Fractone size increases with aging

Fig. 1A and B shows the lateral ventricle of a young and of an aged mouse stained with laminin. The insets show a magnified view of the SVZ, laminin immunoreactivity (laminin-ir) blood vessel basement membranes (arrows) and punctate fractones