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Fibrin deposited in the Alzheimer's disease brain promotes neuronal degeneration

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia and has no effective treatment. Besides the well-known pathologic characteristics, this disease also has a vascular component, and substantial evidence shows increased thrombosis as well as a critical role for fibrin(ogen) in AD. This molecule has been implicated in neuroinflammation, neurovascular damage, blood-brain barrier permeability, vascular amyloid deposition, and memory deficits that are observed in AD. Here, we present evidence demonstrating that fibrin deposition increases in the AD brain and correlates with the degree of pathology. Moreover, we show that fibrin(ogen) is present in areas of dystrophic neurites and that a modest decrease in fibrinogen levels improves neuronal health and ameliorates amyloid pathology in the subiculum of AD mice. Our results further characterize the important role of fibrin(ogen) in this disease and support the design of therapeutic strategies aimed at blocking the interaction between fibrinogen and amyloid-β (Aβ) and/or normalizing the increased thrombosis present in AD.

1. Introduction

Alzheimer's disease (AD) is a multifactorial and severe neurodegenerative disorder for which there is no effective treatment available (Huang and Mucke, 2012). The 2009 World Alzheimer Report estimated that 35.6 million people worldwide were affected by dementia in 2010 and predicted more than 100 million people by 2050 (Prince and Jackson, 2009). Therefore, new therapeutic approaches are sorely needed. This disorder has brain pathologic hallmarks such as amyloid-β (Aβ) plaques and neurofibrillary tangles (Selkoe, 2011) and is characterized by a progressive reduction in cortical thickness and an overall decrease in brain volume with a loss of neurons (Duyckaerts et al., 2009; Gomez-Isla et al., 1996) and synapses (Terry et al., 1991). Besides the strong correlation with different vascular risk factors such as atherosclerosis, hypertension, hypercholesterolemia, and diabetes (de la Torre, 2002; Humpel, 2011), AD pathogenesis also involves cerebrovascular abnormalities such as alterations to the neurovascular unit (Iadecola, 2010) and decreases in cerebral blood flow (Austin et al., 2011; Mazza et al., 2011), suggesting that vascular disease influences AD pathogenesis (Kalaria et al., 2012).

Fibrinogen is a plasma glycoprotein that circulates at high concentration in the blood and is essential for coagulation as it is converted into fibrin in response to injury (Weisel, 2005). The balance between clot formation and degradation needs to be tightly regulated because alterations in this system can induce and exacerbate pathologic situations. Substantial evidence indicates a key role for fibrinogen and fibrin clot formation in AD pathogenesis. Increased fibrin(ogen) deposition is present in the brain parenchyma and brain vessels of human AD patients (Cortes-Canteli et al., 2010, 2012; Cullen et al., 2005; Fiala et al., 2002; Lipinski and Sajdel-Sulkowska, 2006; Ryu and McLarnon, 2009; Viggars et al., 2011) and mouse models of AD (Cortes-Canteli et al., 2010; Paul et al., 2007). However, most of these studies involved the immunohistochemical analysis of fibrin(ogen) in the AD brain using antibodies that fail to distinguish fibrinogen and fibrin (hence the use of the term fibrin(ogen)), making it impossible to know whether the deposits are composed of one or the other, or a mixture of the two. Fibrin(ogen) co-localizes with Aβ in the AD brain (Cortes-Canteli et al., 2010, 2012; Jantaratnotai et al., 2010; Paul et al., 2007; Ryu and McLarnon, 2009), strongly interacts with this peptide (Ahn et al., 2010), and makes fibrin clots more difficult to degrade (Cortes-Canteli et al., 2010; Zamolodchikov and Strickland, 2012). AD mice are at high risk of arterial thrombosis (Jarre et al., 2014), and evidence indicates that there is increased obstruction of the cerebral blood vessels in the AD brain, which could strongly affect overall cerebral circulation. For example, aged ArcAβ AD mice have

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increased occlusion of functional intracortical microvessels (Klohs et al., 2012). Similarly, TgCRND8 AD mice show evidence of increased clotting in their brains, and these fibrin clots are resistant to fibrinolysis (Cortes-Canteli et al., 2010). A prothrombotic state in AD patients is evidenced not only by increased clot formation but also by decreased fibrinolysis and elevated levels of activated coagulation factors and platelets (Cortes-Canteli et al., 2012). Indeed, reducing fibrinogen levels has beneficial effects in AD mice, such as decreasing blood-brain barrier permeability (Paul et al., 2007; Ryu and McLarnon, 2009), neurovascular damage (Paul et al., 2007), inflammation (Paul et al., 2007; Ryu and McLarnon, 2009), and cerebral amyloid angiopathy (Cortes-Canteli et al., 2010). This enhancement in vascular function likely improves cerebral blood flow and hence neuronal function and survival, leading to the amelioration of memory deficits observed in AD mice after fibrinogen reduction (Cortes-Canteli et al., 2010). However, no studies have shown direct evidence that the levels of fibrin(ogen) have an effect on neuronal viability and function. Here, we demonstrate that fibrin(ogen) is present in areas packed with dystrophic neurites and plays a key role in neuronal viability, because decreasing fibrinogen levels reduces the amount of neuronal loss, synaptic dysfunction, and amyloid pathology present in AD mice. We also report that insoluble fibrin accumulates in human and mouse AD brains and correlates with the degree of pathology. These results further characterize the role of fibrin(ogen) in AD pathophysiology and support the design of therapeutic strategies aimed at normalizing the irregular clotting observed in AD.

2. Methods

2.1. Mice

TgCRND8 mice express a double mutant form of the amyloid precursor protein 695 (KM670/671NL + V717F) (Chishti et al., 2001). These mice are on a mixed background (C57xCH3/C57) and develop age-dependent Aβ pathology and memory deficits (provided by Drs M.A. Chishti and D. Westaway, University of Toronto, Canada). Four-, 15-, 58-, and 82-week-old TgCRND8 mice and their wild-type littermates (n = 3–7 mice/group) were thoroughly perfused with saline heparin. Brains were removed, and one hemisphere was embedded, frozen in OCT, and processed for triple immunofluorescence analysis, whereas the cortex and hippocampus of the other hemisphere were dissected out and frozen for subsequent fibrin extraction.

Mice heterozygous for the *fibrinogen Aα chain* ($fbg^{+/-}$) (Suh et al., 1995) were crossed with TgCRND8 mice. TgCRND8; $fbg^{+/-}$ mice and their littermate controls were thoroughly perfused with saline heparin, and brains were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, frozen, and processed for NeuN, lysosomal-associated membrane protein-1 (LAMP-1), and Congo red determination.

All mice were genotyped twice, at time of weaning and at sacrifice. Mice were housed at The Rockefeller University's Comparative Biosciences Center and treated in accordance with IACUC-approved protocols.

2.2. Human samples

Human postmortem tissue was obtained from the Harvard Brain Tissue Resource Center. Blocks of frozen tissue from the superior frontal cortex (n = 4 control and 15 AD cases), the anterior hippocampus with entorhinal cortex (n = 4 control and 16 AD cases), and the hippocampal formation with parahippocampal gyrus (n = 8 control and 29 AD cases) were sliced by cryostat (10-μm sections) for subsequent immunohistochemical analysis. Several sections were also collected in an Eppendorf tube for subsequent fibrin determination.

2.3. Fibrin extraction and Western blot

Mouse and human frozen tissue was homogenized in 5 volumes (g:mL) of phosphate-buffered saline (PBS) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Roche). The homogenate was centrifuged at 4 °C at 10,000*g* for 10 minutes, and the supernatant (soluble fraction) was transferred to a different tube. After several rounds of extraction, the insoluble (fibrin-containing) fraction was extracted as in Tabrizi et al. (1999) with slight modifications. Briefly, the pellet was homogenized in 3 M urea, vortexed for 2 hours at 37 °C, and centrifuged at 14,000*g* for 15 minutes. The supernatant was collected in a different tube, and the pellet was resuspended and vortexed at 65 °C for 30 minutes in reducing SDS loading buffer. Equal amounts were run on a 4%–20% gradient polyacrylamide Criterion gel (Bio-Rad), transferred to a polyvinylidene fluoride membrane (Pall), and incubated with the following antibodies: rabbit polyclonal anti-fibrin(ogen) antibody (gift from Dr J. L. Degen, Cincinnati, OH, USA), mouse monoclonal anti-fibrin antibody (59D8; (Hui et al., 1983), gift from Dr T. Renne, Karolinska Institutet, Sweden), mouse monoclonal anti-Aβ antibody (6E10, Covance), and rat monoclonal anti-tubulin antibody (YOL1/34, Abcam). Tubulin was used as loading control because it is present in different fractions after sequential solubilization steps and extensive rounds of extraction in the rat brain (Schindler et al., 2006). In vitro human or mouse fibrin clots were prepared as positive controls (Cortes-Canteli et al., 2010; Zamolodchikov and Strickland, 2012) and run in parallel with the samples. Samples were subjected to Western blot analysis 4–5 different times. Fibrin β-chain and tubulin bands were quantified using NIH Image J 1.46o software, and the ratio of fibrin:tubulin was plotted on a graph.

2.4. Human brain staining

Frozen human AD and control brain sections (10 μm) were fixed in 4% paraformaldehyde and treated with proteinase K (Dako) before performing the following staining protocols.

Fibrin immunohistochemistry: sections were immersed in methanol/H_2O_2 to inactivate endogenous peroxidases, blocked in Tris buffer with 2% donkey:horse serum (1:1), and incubated overnight with the mouse monoclonal antibody 59D8 that specifically detects human fibrin (Hui et al., 1983). The following morning, sections were incubated with a biotinylated horse anti-mouse antibody, amplified by the VECTASTAIN Elite ABC Ready-to-Use Reagent, and developed using ImmPACT DAB Peroxidase Substrate (all from Vector Laboratories). Sections were then dehydrated, mounted, and imaged using a Zeiss Axiovert 200 microscope.

Triple immunofluorescence: sections were blocked in Tris buffer with 2% goat serum followed by overnight incubation with a mouse monoclonal anti-human LAMP-1 antibody (clone H4A3, Developmental Studies Hybridoma Bank) and a rabbit polyclonal anti-human fibrinogen antibody (Dako). Then, the sections were incubated for 1 hour at RT with the highly cross-adsorbed secondary fluorescent antibodies CF405M goat anti-rabbit and CF555 goat anti-mouse (Biotium), rinsed, and incubated overnight with anti-Aβ monoclonal antibody 6E10 labeled with Alexa Fluor 488 (Covance). The tissue was incubated with 0.3 % Sudan Black B in 70% ethanol to block lipofuscin autofluorescence and finally covered with Vectashield (Vector Laboratories). Secondary controls omitting primary antibodies as well as controls using each individual primary antibody alone were carried out in parallel. An inverted TCS SP8 laser scanning