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Reserach Paper

Increased expression of toll-like receptor 3, an anti-viral signaling molecule, and related genes in Alzheimer's disease brains

Douglas G. Walker[a,b,c,*], Tiffany M. Tang[b,1], Lih-Fen Lue[b,c]

[a] Molecular Neuroscience Research Center, Shiga University of Medical Science, Otsu, Japan
[b] Neurodegenerative Disease Research Center, Biodesign Institute, Arizona State University, Tempe, Arizona, USA
[c] Banner Sun Health Research Institute, Sun City, Arizona, USA

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ABSTRACT

The focus of this study is the expression of Toll-like receptor-3 (TLR-3), a receptor for double-stranded RNA, in human brains affected by Alzheimer's disease (AD) pathology. Toll-like receptors are a family of pattern recognition molecules primarily involved in host defenses to microbial pathogens, but roles in neurodegenerative disease have also been shown, as amyloid beta (Aβ) can be a ligand for TLR-2 and -4 and α-synuclein for TLR-1 and TLR-2, while TLR-9 activation promotes Aβ removal. However, involvement of TLR-3 in AD has not been rigorously studied. Immunohistochemical analyses in human temporal cortical sections with a validated antibody for TLR-3 predominantly identified microglia, particularly strongly in cells associated with amyloid plaques, also brain vascular endothelial cells and subsets of astrocytes, but not neurons or p62-immunoreactive structures. Microglial TLR-3 colocalized with the endosomal/lysosomal marker CD68, which identifies phagocytic cells. Quantitative analyses of neuropathologically-staged human brain middle temporal gyrus samples using immunohistochemistry and mRNA expression methods demonstrated increased TLR-3 immunoreactivity and increased TLR-3 mRNA in AD compared to non-demented cases. There were significant positive correlations between TLR-3 mRNA levels and plaque or tangle loads in both series of samples. Increased expression of interferon beta (IFN-β) and interferon regulatory factor (IRF)-3 mRNA, two factors induced by TLR-3 signaling, were detected in the AD cases. Increased expression of TLR-4 and TLR-9 mRNA was also observed in these same samples, but not TLR-2. *In vitro* cultured human brain microglia responses to Aβ inflammatory activation were not altered by TLR-3 activation with activator polyinosinic;polycytidylic acid (poly I:C), while human brain endothelial cells showed reduction in responses when stimulated with both agents. Treatment of microglia with poly I:C did not increase their uptake and breakdown of Aβ.

1. Introduction

Toll-like receptors (TLRs) in humans are a family of 10 pathogen-associated pattern recognition receptors mainly involved in host immunity to microbial pathogens and recognize different viral, bacterial and fungal nucleic acids, lipids or lipoproteins (O'Neill et al., 2013). Additional TLR ligands associated with cellular damage/death in the absence of infection have been identified (Vidya et al., 2017). TLRs are type I transmembrane receptors localized to plasma membranes or endolysosomal membranes with conserved molecular structures of ectodomains containing leucine-rich repeats. TLR-1, TLR-2, TLR-4, TLR-5, TLR-6 and TLR-10 are primarily localized to the plasma membrane, while TLR-3, TLR-7, TLR-8 and TLR-9 are mainly on intracellular endolysosomal vesicles. In addition to responding to microbial components, TLR responses to molecules associated with dying cells, such as oxidized low-density lipoprotein, oxidized phospholipids, β-defensin-2, high-mobility group box 1, degradation products of extracellular matrix and heat shock proteins, and cellular RNA and DNA amongst others, have been shown (Leifer and Medvedev, 2016). Several TLRs can be activated by abnormal molecules associated with neurodegeneration, including TLR-2 and TLR-4 by amyloid beta (Aβ) (Balducci et al., 2017; Reed-Geaghan et al., 2009) and TLR-1 and TLR-2 by α-synuclein (Daniele et al., 2015; Kim et al., 2016). Activation of TLR-9, an endosome localized TLR, by its ligand unmethylated DNA has been shown to stimulate Aβ removal in different Alzheimer's disease (AD) animal models (Scholtzova et al., 2009, 2014, 2017). It has been hypothesized that TLR activation might enhance activity of phagocytic cells through stimulation of autophagy processes (Xu et al., 2008; Zhan

* Corresponding author at: Molecular Neuroscience Research Center, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga 520-2192, Japan.
E-mail address: walker@belle.shiga-med.ac.jp (D.G. Walker).
[1] **Current address:** Biomedical Sciences Graduate Program, College of Medicine, Pennsylvania State University, Hershey, PA, U.S.A.

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Table 1
Demographic details of human brain cases used.

Disease state (n)	Age	Sex	PMI	ApoE4	Plaques	Tangles
A: Middle temporal gyrus (Immunohistochemistry)						
ND-LP (n = 15)	86.1 ± 6.7	10 M/5F	2.7 ± 0.7	10%	2.1 ± 2.1	3.7 ± 2.1
ND-HP (n = 10)	84.6 + 6.0	7 M/3F	3.0 ± 0.6	25%	9.1 + 1.7	2.9 ± 1.1
AD (n = 11)	85.8 + 5.6	6 M/5F	2.6 ± 0.4	36%	14.0 ± 1.2	12.2 ± 2.7
B: Middle temporal gyrus – first series (RNA expression)						
ND (*n* = 19)	79.5 ± 11.5	15 M/4F	3.0 ± 1.1	5.5%	0.5 ± 1.3	3.4 ± 2.8
AD (n = 24)	84.1 + 6.2	12 M/12F	3.1 ± 0.9	18%	13.8 ± 1.8	13.2 ± 3.0
C: Middle temporal gyrus – second series (RNA expression)						
ND-LP (*n* = 14)	85.4 ± 9.0	7 M/7F	3.1 ± 1.0	4%	1.1 ± 1.8	5.3 ± 2.4
ND-HP (n = 13)	87.3 + 7.1	6 M/7F	2.7 ± 0.3	11.5%	11.4 + 1.9	5.1 + 2.0
AD (n = 15)	79.7 + 4.6	10 M/5F	3.5 ± 0.6	30%	14.3 ± 0.8	13.4 ± 2.4

Abbreviations: PMI: post-mortem interval ± standard error of mean (SEM) (hr); ApoE4: % ApoE4 alleles; Plaques: Mean plaque score ± SEM (0–15); Tangles: Mean tangle score ± SEM (0–15); ND: non-demented; AD: Alzheimer's disease; ND-LP: non-demented low plaque; ND-HP: non-demented high plaque.

et al., 2014). Colocalization of TLR-3 and autophagy markers was identified in thalamus neurons of preterm infants with white matter injury (Vontell et al., 2015).

Complex signaling pathways activated by TLRs have been defined, which result in inflammatory activation through the transcription factor NFκB or through activation of type 1 interferon (IFN) responses (Kawai and Akira, 2007a, 2007b; Takeuchi et al., 2004). All TLRs except TLR-3 use the adaptor protein myeloid differentiation response protein 88 (MyD88) as a signaling intermediate. By contrast, TLR-3, an endosomal-located receptor, whose native ligand is double-stranded RNA (dsRNA) primarily utilizes Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) as an adaptor protein to activate signaling. TLR-3 activation is involved in cellular responses to a number of different viruses, including infection by the DNA herpes simplex virus, which results in a strong type 1 interferon antiviral response (Boivin et al., 2008; Majde et al., 2010; Nazmi et al., 2014; Wang et al., 2004). Although discounted for many years, recent studies have provided new evidence of association of viruses, including human herpesvirus (HHV)-6A and -7 and hepatitis B virus, with AD pathology (Mastroeni et al., 2018; Readhead et al., 2018). Recent results showed that TLR-3 can be activated by different mRNA species (Kariko et al., 2004b), and even double stranded siRNA complexes (Kariko et al., 2004a). A unique non-nucleic acid ligand for TLR-3 was identified as stathmin, a microtubule-associated regulator cytoskeleton protein (Bsibsi et al., 2010). Stathmin is abundant in brain and was shown to activate TLR-3 signaling in human astrocytes and microglia in a similar manner to the artificial dsRNA ligand polyinosinic;polycytidylic acid (poly I:C).

Controlling innate inflammation due to enhanced TLR activation has been a therapeutic target for a number of diseases; however, with regards to TLR-3, there have been studies showing that enhanced TLR-3 signaling can have protective effects (Li et al., 2015; Zhou et al., 2015). The possible involvement of TLR-3 and type 1 beta interferon (IFN-β) responses in neuronal autophagy was demonstrated in PD animal models. Lack of IFN-β directly resulted in neurodegeneration and accumulation of α-synuclein in Lewy body like structures due to defects in autophagy (Ejlerskov et al., 2015).

TLR-3 expression has been demonstrated in many different cell types, but particularly in inflammatory cells with significantly increased expression in mature dendritic myeloid cells (Muzio et al., 2000). The increased expression of TLR-3 in monocyte-derived dendritic cells (DC) defined it as a marker for this cell type (Muzio et al., 2000). In brain, expression has been observed in microglia, astrocytes, endothelial cells and neurons (Bsibsi et al., 2006, 2002; Facci et al., 2014; Jack et al., 2007; Jeong et al., 2015; Li et al., 2013; Nagyoszi et al., 2010). A recent study showed that TLR-3 activation in neurons affected neuronal morphology and expression of genes involved in schizophrenia (Chen et al., 2017). Other studies have shown TLR-3 activation was involved in neurogenesis (Lathia et al., 2008; Okun et al., 2010). A recent review of TLRs in AD succinctly summarized roles for TLR-2, TLR-4 and TLR-9, but there have been no human brain tissue studies of TLR-3 involvement in AD neurodegeneration (Su et al., 2016)

Studies involving TLR-3 activation, primarily using poly I:C as ligand, have shown both damaging and protective effects. Direct injection of poly I:C into brain resulted in significant neurotoxicity in the substantia nigra through inflammatory activation (Deleidi et al., 2010). Similarly, intravenous administration of poly I:C to AD model mice increased Aβ deposition and phosphorylated tau pathology (Krstic et al., 2012). Treatment of astrocytes and microglia with poly I:C resulted in increased expression of pro-inflammatory and anti-inflammatory cytokines depending on dose (Bsibsi et al., 2010; Bsibsi et al., 2006), including a number of neuroprotective cytokines and growth factors. Involvement of TLR-3 signaling in the absence of virus infection has been established, and protective effects of enhanced TLR-3 signaling in cerebral ischemia and possibly multiple sclerosis have been identified (Pan et al., 2012; Shi et al., 2013; Wang et al., 2014).

The purpose of the studies in this report was to assess whether there might be involvement of TLR-3 in AD-related inflammatory pathology. The first stage was to identify cellular localization and gene expression in AD brains, and then to model findings using unique *in vitro* cellular models of microglia and brain endothelial cells derived from human aged brains. We identified increased expression of TLR-3 associated with increasing amounts of AD pathology, but the changes in expression appeared to be late in the disease process. *In vitro* experiments showed that Aβ treatment did not induce TLR-3 expression by microglia.

2. Materials and methods

2.1. Human brain tissue samples and diagnoses criteria

Human brain tissue samples used in this study were obtained from the Banner Sun Health Research Institute Brain and Body Donation Program (Beach et al., 2008, 2015). The operations of the Brain and Body Donation Program have been reviewed by an Institutional Review Board (Western IRB, Puyallup, WA). Summary of details of all cases used in this study are summarized in Table 1. The use of human tissues for experimentation had the approval of all institutions involved.

To assess severity of AD pathology in each case, tissue sections from 5 brain regions (entorhinal cortex, hippocampus, frontal cortex, temporal cortex and parietal cortex) were stained with Thioflavin S, Gallyas or Campbell-Switzer histological stains and assessed semi-quantitatively for the density of neurofibrillary tangles and amyloid plaques. Each brain region was ranked on a scale of 0–3. By combining the measures across these 5 brain regions, assessment of AD pathology was ranked on a non-parametric scale of 0–15 for plaques and tangles (Dugger et al., 2012). Two sets of cases were subdivided into non-