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Blockage of CR1 prevents activation of rodent microglia

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ABSTRACT

The importance of the complement system in Alzheimer's disease (AD) pathogenesis has been emphasized through recent genome wide association studies. However, the cellular and molecular role of these complement proteins is not fully understood. Microglia express complement receptors and the activation of specific receptors may increase Aβ clearance and reduce neurodegeneration. Here, we investigated the contribution of complement receptor 1 (CR1), the second most significant hit in GWAS studies, on microglia to neuronal damage. We show that microglia displaying an activated phenotype demonstrate an increase in CR1 expression. We also provide evidence that activation of microglial CR1 was detrimental to neurons and this correlated with an increase in microglial intracellular superoxide generation, and tumour necrosis factor-α (TNFα) and interleukin-1 β (IL-1β) secretion. Amyloid-β 42 ($A\beta_{1-42}$)-treated microglia displayed an increased ability to phagocytose dextran beads following antibody blockage of CR1 but a decreased capacity to phagocytose fluorescent-tagged $A\beta_{1-42}$. Together, these results indicate that microglial CR1 plays a role in the neuronal death observed in AD and investigating this further may provide a possible strategy to control neurotoxicity in the AD brain.

Introduction

Recent genome wide association studies (GWAS) in Alzheimer's disease have highlighted the importance of the complement cascade in the pathogenesis of Alzheimer's disease (Brouwers et al., 2011; Crehan et al., 2012; Jones et al., 2010; Lambert et al., 2009). This work confirms the pathogenic relevance of earlier work implicating this cascade in the pathology of the disease (McGeer and McGeer., 2002; McGeer et al., 1989) and work showing that Aβ/amyloid deposits activate the complement cascade (Guerreiro et al., 2012; Rogers et al., 1992; Velazquez et al., 1997).

The second most significant hit in these GWAS, after apolipoprotein E, was the CR1 gene which encodes complement receptor 1, a complement regulator and receptor for C3b (Hollingworth et al., 2011; Naj et al., 2011). Based on this analysis, we investigated the relationship between Aβ and CR1, by using blocking CR1 antibody (Anti-CD35) and small interfering RNA (siRNA) to assess whether this relationship could underpin the association with disease.

The association observed between the CR1 loci and age-related cognitive decline and plaque burden has been implicated to have connections to impaired clearance of Aβ plaques in the brain of AD patients (Chibnik et al., 2011). Although the recruitment of phagocytes and inflammatory mediators is intended to be beneficial, under certain conditions, these processes can prove harmful instead. Classical markers of immune-mediated damage have been identified in Alzheimer's disease (AD) brains including major histocompatibility complex class I and II positive microglia (McGeer et al., 1987; Perlmutter et al., 1992; Tooyama et al., 1990).

In normal brain, microglia become reactive, surround damaged or dead cells and clear cellular waste from the area to promote regeneration and repair (Fetler and Amigorena, 2005). Microglia generally have beneficial effects, but their overstimulation can promote neurotoxicity due to pathogenic signals, including Aβ, resulting in the production of excess free radicals, pro-inflammatory cytokines, complement proteins and glutamate (Morales et al., 2010). In AD, microglia display an early reactive phenotype (Lautner et al., 2011), and changes in the immune response are another risk marker for the development of AD (Jones et al., 2010).

The expression and distribution of CR1 in humans and rodents contrast because human CR1 is encoded by a separate gene to human CR2,

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; Apo, apocynin; APP, amyloid precursor protein; CGC, cerebellar granule cells; CR1, complement receptor 1; DIV, days in vitro; DHE, dihydroethidium; D-MEM, Dulbecco's modified eagle medium; EA, ethacrynic acid; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; GWAS, genome-wide association study; IL-1β, interleukin-1 beta; iNOS, inducible nitric oxide synthase; LOAD, late-onset Alzheimer's disease; LPS, lipopolysaccharide; MCB, monochlorobimane; MGCM, microglial conditioned medium; NGS, normal goat serum; NO, nitric oxide; $O^{\bullet -}$, superoxide; PBS, phosphate buffered saline; PFA, paraformaldehyde; PI, propidium iodide; PMA, phorbol myristate acetate; PVDF, polyvinylidene difluoride; RAGE, receptor for advanced glycation end product; ROS, reactive oxygen species; SDS-PAGE, sodium sulphate polyacrylamide gel electrophoresis; TNFα, tumour necrosis factor-alpha.

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but murine CR2 encodes both CR1 and CR2 (Kurtz et al., 1990; Molina et al., 1990). However the protein sequence and function of the murine specific gene, CR1-related protein Y (Crry), display more likeness to human CR1 than mouse CR1 (Killick et al., 2012). In view of this relationship, we confirmed our findings using a CR1 blocking antibody with siRNA to downregulate Crry.

Exposure of microglia to extrinsic C1q complement protein demonstrated a shift towards a pro-inflammatory phenotype, similar to that seen after the exposure of microglia to LPS, with a release in interleukin-6, tumour necrosis factor-α (TNFα), nitric oxide and an oxidative burst (Färber et al., 2009). Here we investigated how modulation of CR1 signalling influenced the microglial phenotype during exposure of activators complicit in microglial responses in AD. In this work we show that blocking microglial CR1 has a positive effect on neuronal survival. We provide evidence that microglial CR1 can elicit a neurotoxic effect which correlated with enhanced cytokine and superoxide production, but that blockage of CR1 does not contribute a protective response by triggering glutathione production or inhibiting iNOS induction. We also demonstrated an increased ability of $A\beta_{1-42}$ treated microglia to phagocytose dextran beads following antibody blockage of CR1 but a reduced ability to phagocytosis fluorescent $A\beta_{1-42}$.

Materials and methods

Animals and materials

Sprague Dawley rats were bred and reared in house from stock animals from Charles River UK Ltd. (Kent, UK). Tissue culture components were from Invitrogen (Paisley, UK). Anti-CD35 functional blocking antibody, C3b receptor was from antibodies-online.com (Atlanta, USA), anti-iNOS was from BD Biosciences (Oxford, UK), anti-CD35 antibodies were from Hycult biotech, and antibodies were raised against purified human C3bR (Uden, the Netherlands, clone 31R) and Abcam (Cambridge, UK, clone E11), and details of the immunogen for this antibody are not available. Anti-NeuN antibody was from Merck Millipore (Oxfordshire, UK), cleaved caspase-3 antibody was from Cell Signalling Technology (MA, USA), dextran beads were from Molecular Probes, Invitrogen (Paisley, UK), β-amyloid (1–42) HiLyte Fluor™ 488-labeled was from AnaSpec (Fremont, CA, USA), Vectashield mountant for fluorescence was from Vector (Peterborough, UK), and enhanced chemiluminescence reagent (ECL) was from Amersham Pharmacia (Buckinghamshire, UK). ON-TARGETplus Crry siRNA and Cyclophilin B control siRNA was purchased from Thermo-scientific (CO, USA). Quantikine Rat TNFα and IL-1β Immunoassay kits were from R&D Systems (Abingdon, UK). Anti-β-actin and all other reagents were from Sigma (Dorset, UK).

Cell culture preparation and treatment

Animals (5 day old Sprague Dawley rat pups) were sacrificed through cervical dislocation and decapitation in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom). Cerebellar granular cell (CGC) neuronal–glial cultures were prepared as described (Piers et al., 2011). These cultures contain approximately 5–10% of microglia per 100 neurons as determined by Isolectin B4 staining and NeuN staining (Pocock, unpublished observations), a ratio representative of in vivo data. Mixed cultures of hippocampal neurons and glial cells were prepared as described previously with modifications, from Sprague–Dawley rat pups 2–4 days post-partum (Vaarmann et al., 2010). Hippocampi and cortex were removed into ice-cold phosphate buffered saline (PBS). The tissue was minced and trypsinised (0.25% for 5 min at 37 °C), triturated and plated on poly-D-lysine-coated coverslips and cultured in Dulbecco's modified eagle medium (D-MEM) (GIBCO, Carlsbad, CA) supplemented with 10% foetal bovine serum (FBS), penicillin and streptomycin (GIBCO, Carlsbad, CA).

Cultures were maintained at 37 °C at 5% CO_2 and were viable for experimentation at 7 days in vivo (DIV).

Microglial cells were isolated from the brains of 5 day old Sprague Dawley rat pups using Percoll gradients as previously described (Hooper et al., 2009a). The BV2 microglial cell line was a kind gift from Dr Claudie Hooper (MRC Centre for Neurodegenerative Research, Institute of Psychiatry, Kings College London, UK) and was originally obtained from Dr FS Tzeng (Department of Life Sciences, National Cheng Kung University, Taiwan). Where indicated in the figure legend, CGCs, hippocampal neuronal–glial cultures, microglia or BV2s were treated directly with LPS (1 μg/mL) or $A\beta_{1-42}$ (20 nM) for 24 h or 48 h with or without the pre-treatment with CR1 blocking antibody (2 μg/mL).

Neurons were also exposed to microglial conditioned medium (MGCM) collected from primary microglia following 24 h treatment and added to CGCs in a ratio of 1:1 with the original CGC medium and the neurons incubated for a further 48 h, as previously described (Davenport et al., 2010).

Phagocytic assay

Treated microglia were incubated with 10 kDa TRITC-conjugated dextran beads (20 μg/mL) for 3 h or with β-Amyloid (1–42) HiLyte Fluor™ 488-labeled (5 μg/mL) for 2 h at 37 °C. Cells were then incubated with 5 μg/mL of the nuclear stain 2′-[epoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazol Hoechst 33342 (Hoechst 33342) for 10 min and then washed in PBS to remove loose beads and bead uptake was visualized by fluorescence microscopy. Fluorescence images were captured using TRITC and FITC filter sets on a Zeiss fluorescence microscope plus 40× Neofluor objective (Zeiss Axioskop 2, Oberkochen, Germany). Data were collected from at least 3 fields of view per coverslip with each condition repeated on 3 coverslips in 3 separate experiments. Labelled cells were counted as a percentage of total cells per field. Control cells were incubated with beads alone at 37 °C for all conditions or at 4 °C, the latter as a binding control.

Assessment of apoptosis and cell death

Treated CGCs were incubated with 5 μg/mL Hoechst 33342 for 20 min and 5 μg/mL propidium iodide (PI) for 30 min as previously described (Pinteaux-Jones et al., 2008). Apoptotic cells, which show bright blue pyknotic nuclei, and necrotic cells, which display red nuclei, were observed using DAPI (blue fluorochrome 364 nm) and TRITC (red fluorochrome, 543 nm) filters, respectively, on a Zeiss fluorescence microscope plus 40× Neofluor objective (Zeiss Axioskop 2, Oberkochen, Germany). Cell counts were performed on a minimum of 3 fields per coverslip, 3 coverslips per treatment from 3 independent experiments. Treated hippocampal cells were fixed and stained for NeuN (see below) to assess neuronal loss as NeuN does not stain dead cells.

Immunocytochemistry

Treated microglia or hippocampal neuronal–glial cultures were fixed with 4% paraformaldehyde (PFA), permeabilised with 100% ice-cold methanol or 0.1% Triton-X100 and non-specific binding blocked with 4% NGS. Cultures were incubated at 4 °C overnight with primary antibody, NeuN (1:500) or cleaved caspase-3 (1:500) followed by incubation at room temperature with the appropriate secondary antibody for 2 h. Finally cells were counterstained with DAPI, mounted with Vectashield and visualized by fluorescence microscopy.

Superoxide and glutathione live cell imaging

Treated primary microglia or BV2 microglia were assessed for superoxide ($O_2^{\bullet-}$) production by incubation at 37 °C for 40 min with dihydroethidium (DHE) (5 μM) and were counterstained with Hoechst 33342 as previously described (Mead et al., 2012). Duplicate conditions were treated with apocynin (10 mM) to verify $O_2^{\bullet-}$ production was via