Biochimica et Biophysica Acta 1842 (2014) 107–115

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Complement inhibition and statins prevent fetal brain cortical abnormalities in a mouse model of preterm birth

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ARTICLE INFO

Article history:
Received 24 June 2013
Received in revised form 17 October 2013
Accepted 22 October 2013
Available online 30 October 2013

Keywords:
Preterm birth
Cortical fetal brain damage
Complement
Glutamate
Statin

ABSTRACT

Premature babies are particularly vulnerable to brain injury. In this study we focus on cortical brain damage associated with long-term cognitive, behavioral, attentional or socialization deficits in children born preterm. Using a mouse model of preterm birth (PTB), we demonstrated that complement component C5a contributes to fetal cortical brain injury. Disruption of cortical dendritic and axonal cytoarchitecture was observed in PTB-mice. Fetuses deficient in C5aR (−/−) did not show cortical brain damage. Treatment with antibody anti-C5, that prevents generation of C5a, also prevented cortical fetal brain injury in PTB-mice. C5a also showed a detrimental effect on fetal cortical neuron development and survival in vitro. Increased glutamate release was observed in cortical neurons in culture exposed to C5a. Blockade of C5aR prevented glutamate increase and restored neurons dendritic and axonal growth and survival. Similarly, increased glutamate levels – measured by ^{1}HMRS – were observed in vivo in PTB-fetuses compared to age-matched controls. The blockade of glutamate receptors prevented C5a-induced abnormal growth and increased cell death in isolated fetal cortical neurons. Simvastatin and pravastatin prevented cortical fetal brain developmental and metabolic abnormalities -in vivo and in vitro. Neuroprotective effects of statins were mediated by Akt/PKB signaling pathways. This study shows that complement activation plays a crucial role in cortical fetal brain injury in PTL and suggests that complement inhibitors and statins might be good therapeutic options to improve neonatal outcomes in preterm birth.

1. Introduction

Preterm birth is an important cause of perinatal mortality and morbidity. Between 23 and 32 weeks of pregnancy, the human fetal brain is at a critical stage of development and vulnerable to injury. 25 to 50% of infants that were born prematurely experience long-term cognitive, behavioral, attentional or socialization deficits [1–3]. MRI studies in preterm infants showed decreased volume in the cerebral cortex, suggesting a role for the cortex in these long term complications [1]. Unfortunately, the mechanisms behind these long term disabilities are still unclear, preventing therefore the development of diagnostic methods and therapies.

During pregnancy, the developing brain is particularly susceptible to ischaemic and inflammatory insults, the latter often due to maternal intrauterine inflammation/infection. Despite the growing association between inflammation with fetal brain injury [4–6], the mediators and receptors involved remain unknown.

Using a mouse model of inflammation-induced PTB [7,8], we demonstrated that complement activation, in particular C5a, plays a crucial role in the cervical ripening that leads to PTB [7]. Here, we investigate if C5a also causes damage in the developing fetal brain cortex in this model. We focused on the cerebral cortex because cognitive deficits are by far the dominant neurodevelopmental sequelae in infants born preterm.

In an attempt to find a treatment to prevent fetal cortical brain damage we studied the effects of complement inhibition in this model. Furthermore, we hypothesized that statins which prevented complement-mediated tissue injury in several animal models [9–11], would also prevent fetal cortical brain developmental abnormalities.

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http://dx.doi.org/10.1016/j.bbadis.2013.10.011

2. Material and methods

2.1. Animals

All housing and experimental procedures were performed in compliance with the UK Home Office Animals Scientific Procedures Act 1986 (Home Office project licence number 60/4305).

C57BL/6 from commercial vendors and C5a receptor (C5aR)-deficient mice, generated by homologous recombination technology, by Dr. Craig Gerard (Harvard Medical School) [12] were used in all the experiments. It has been described that subclinical genital tract infections are often associated with spontaneous preterm birth in women. Thus, we used a mouse model of spontaneous PTD which resembles this clinical scenario [4]. In this model, mice received LPS (*Escherichia coli* serotype 055:B5) (250 μg/mouse) intravaginally on day 15 of pregnancy [4].

This dose showed to induce cervical ripening [4] but not systemic disease in the mother. It has been documented that the vagina is a poor route for systemic delivery primarily due to the poor absorption across the vaginal epithelium [13].

To determine whether LPS administered through the vaginal canal ascends to the uterus and/or reaches systemic circulation, a group of mice were administered intravaginal LPS labeled with FITC (Sigma Chemicals, St Louis, MO). FITC-LPS content in the vagina, cervix, uterine horns, amniotic fluid and blood was measured 12 h after LPS administration with a Perkin-Elmer luminescence spectrometer (San Jose, CA, USA). A standard curve was constructed by plotting fluorescence (arbitrary units) against different concentrations of FITC-LPS.

In this model, 100% of the mice deliver preterm before gestational day 17. The pregnant mice treated with LPS were euthanized 18 to 24 h after treatment. The age-matched control mice, that received intravaginal saline, were euthanized at the same time point. Amniotic fluid and fetal brains were collected in the LPS-treated mice and age-matched controls. To inhibit C5, mice were treated on days 10 and 12 of pregnancy with anti-C5 mAb (1 mg, intraperitoneally) [14] or murine IgG as a control and on day 15 they received intravaginal LPS.

A group of mice received pravastatin (10 μg/mouse, i.p.) or simvastatin (20 μg/mouse, i.p.) [9] 24 h before and 2 h after LPS intravaginal administration. Fetal genotypes (C5aR +/− and C5aR −/−) were determined by polymerase chain reaction. The primers for C5aR were obtained from Applied Biosystems (Foster City, CA).

2.2. Simvastatin and pravastatin solutions

Simvastatin (Sigma Chemical) was prepared as a 4 mg/ml stock. Briefly 4 mg of simvastatin was dissolved in 100 μl of ethanol and 150 μl of 0.1 N NaOH and incubated at 50 °C for 2 h, then the pH was adjusted to 7, and the total volume was corrected to 1 ml. The stock solution was diluted to the appropriate concentration in sterile PBS. Pravastatin (Sigma Chemical) was directly dissolved in sterile PBS.

2.3. Measurement of C5a

C5a is rapidly cleaved to the more stable metabolite C5adesArg. Thus, we measured C5adesArg to estimate C5a levels. C5adesArg levels in amniotic fluid and fetal brain cortex was measured by sandwich ELISA as previously described [7,8] using rat anti-mouse C5a and biotin rat anti-mouse C5a (BD Biosciences Pharmingen). The fetal cortical brain tissue was isolated using a dissecting microscope.

2.4. Isolation of fetal cortical neurons

Cortical neurons from fetal brains were isolated as described by Kim and Magrane [15]. After surgical removal of the cerebellum, midbrain and hippocampus the cortical tissue was sequentially incubated with trypsin and DNAse to dissociate the neurons. More than 98% of the cells stained positive for βIII tubulin and negative for GFAP indicating that there is no glial contamination. After centrifugation the dissociated neurons were resuspended in a neuronal culture medium [15] and plated on laminin/polylysine-coated dishes (5×10^5 cells/cm^2). These cells extend their neurites and establish synapses in culture after 10 days in culture and thus represent an accessible model to study cortical brain development and evaluate synapses and neuritic networks in vitro [15]. Using these primary neuronal cultures we investigated the effects of complement component C5a on brain development by evaluating neuritic networks formation in vitro and we tested statins as a therapeutic approach to prevent brain injury. Cortical neurons from day 16 fetuses were isolated and cultured on coverslips coated with laminin/polylysine. After 7 days in culture (7 days in vitro (7DIV)) neurons were exposed to different treatments (C5a (100 nM) (C5a), glutamate (glu) (50 μM), C5aR antagonist peptide (AcPhe[L-ornithine-Pro-D-cyclohexylalanine-Trp-Arg]) (C5aR-AP) (1 μM) [16], MK-810 (1 μM), LY294002 (50 μM) or only media (control)).

The dose of C5a was selected from dose-response studies (Supplemental material). A group of neurons was preincubated with pravastatin (20 μg/ml) or simvastatin (40 μg/ml) 6DIV and on 7DIV C5a was added to the media (P + C5a; S + C5a)). A group of control neurons and a group of neurons preincubated with LY294002 for 12 h were exposed to pravastatin or simvastatin on 6DIV and on 7DIV C5a was added to the media. Formation of neuritic networks in each experimental group was evaluated on 10DIV by immunohistochemistry using BIII tubulin antibodies after fixation with paraformaldehyde 4%. Glutamate release to the media and viability of neurons were measured on 7DIV, 6 h after the respective treatment. Glutamate was measured using a commercial kit (Biovision Incorporated, Milpitas, CA, USA and lactate dehydrogenase (LDH) activity was determined by measuring the linear rate of consumption of NADH absorption (340 nm) during the reduction of pyruvate to lactate using a spectrophotometer. Caspase-3 activity in isolated cortical neurons was measured by a fluorometric assay based on the hydrolysis of acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase 3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) (Sigma Chemicals, St Louis, MO, USA). After each treatment, the cortical neurons were lysed with Tris buffer (50 mol/L Tris–HCL, 1 mmol/L EDTA, and 10 mmol/L EGTA at pH = 7.4) containing 10 μmol/L digitonin for 20 min at 37 °C. The lysates were subjected to a quick centrifugation at 20,000 ×*g* and cell-free supernatants were incubated with Ac-DEVD-AFC, 50 μmol/L) for 1 h at 37 °C. The caspase-3 activity was measured by florescence using a microplate reader with excitation at 360 nm and emission at 460 nm. The caspase-3 activity was expressed as fluorescent units (FUs/mg protein).

2.5. Proton magnetic resonance spectroscopy studies (^{1}HMRS)

This non-invasive in vivo imaging modality was used to study the biochemical and metabolic profile in fetal brain during preterm. All MRI experiments were performed using a 7-T horizontal bore NMR spectrometer (Agilent, Yarnton, UK), equipped with a high-performance gradient insert (12-cm inner diameter), maximum gradient strength 400 mT/m. 18 to 24 h after their respective treatments, the mice were anesthetized with 1.8% isofluorane in oxygen/air (50/50,1 L/min) and placed in a cradle (Rapid Biomedical GmbH, Rimpar, Germany). The rectal temperature and respiration rate were monitored throughout the experiments, and body temperature was maintained at 37 °C with a heat fan. A birdcage coil (33-mm diameter) was used for radio frequency transmission and signal reception.

In order to place the spectroscopy voxel (Fig. 2H) in the brain, good anatomical details are required. To this end, respiration-gated T2-weighted fast spin echo images (echo train length of 4 or 8) of 1 mm slice thickness in 3 orthogonal directions were collected with the following parameters: repetition time (TR) ≈ 2500 ms depending on the respiration rate; effective echo time = 50 ms; field of view = 35 mm × 35 mm; matrix = 192 × 192, 2 signal