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Research Paper

Tumor necrosis factor-inducible gene 6 protein: A novel neuroprotective factor against inflammation-induced developmental brain injury

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ABSTRACT

Inflammation is an important factor contributing to developmental brain injury in preterm infants. Although tumor necrosis factor-inducible gene 6 protein (TSG-6) has immunomodulatory effects in several inflammatory conditions of adult animals, nothing is currently known about the role of TSG-6 in the developing brain, its impact on perinatal inflammation and its therapeutic potential. The aim of the current work was 1) to characterize the developmental expression of TSG-6 in the newborn rat brain, 2) to evaluate the impact of LPS exposure on TSG-6 expression and 3) to assess the therapeutic potential of exogenous TSG-6 administration. Brain hemispheres of healthy Wistar rats (postnatal day 1–postnatal day 15 (P1–P15)) were evaluated with regard to the physiological expression of TSG-6. LPS-treated rats (0.25 mg/kg LPS i.p. on P3) were analyzed for inflammation-induced changes in TSG-6 and cytokine expression. To evaluate whether exogenous recombinant human (rh)TSG-6 affects inflammation-induced brain injury, newborn Wistar rats, exposed to LPS on P3, were treated with rhTSG-6 i.p. (four repetitive doses of 2.25 mg/kg every 12 h, first dose 3 h before LPS injection). PCR, Western blotting and multiplex ELISA were performed according to standard protocols. TSG-6 is physiologically expressed in the developing brain with a linear increase in expression from P1 to P15 at the mRNA level. At P6, regional differences in TSG-6 expression in the cortex, thalamus and striatum were detected at mRNA and protein level. Furthermore, TSG-6 gene expression was significantly increased by inflammation (induced by LPS treatment). Combined treatment with LPS and TSG-6 vs. LPS exposure alone, resulted in significant downregulation of cleaved caspase-3, a marker of apoptosis and neuronal plasticity. In addition, several inflammatory serum markers were decreased after TSG-6 treatment. Finally, TSG-6 is physiologically expressed in the developing brain. Changes of TSG-6 expression associated with inflammation suggest a role of TSG-6 in neuroinflammation. Reduction of cleaved caspase-3 by TSG-6 treatment demonstrates the putative neuroprotective potential of exogenous TSG-6 administration in inflammation-induced developmental brain injury.

1. Background

The report for disability-adjusted life years, based on data from the Global Burden of Disease Study 2010, ranks preterm birth complications at #8 worldwide, which is an improvement compared to #3 in 1990. Statistics for years of life lost show similar results with a change from #3 in 1990 to #7 in 2010 (Murray et al., 2012). Thus, modern therapeutic strategies and social programs, most prominently the millennium development goals, have achieved significant improvements. Nevertheless, the impact of preterm birth complications on global health is still very high and requires further improvements. Even though the rate of preterm births is increasing among neonatal mortalities, overall mortality has declined over the past three decades. But with increasing survival rates of immature neonates, the absolute number of babies suffering morbidity is rising (Wen et al., 2004; Saigal and Doyle, 2008). The organs most affected by prematurity are the brain (Rees and Inder, 2005) and the lung (Jobe and Bancalari, 2001). Most important for long-term prognosis of brain involvement is the Encephalopathy of Prematurity (Volpe, 2009), which comprises two severe neurological disorders: periventricular leukomalacia and neuronal/axonal disease. Long-term sequels are motoric deficits up to cerebral palsy, sensory impairment of the auditory and visual system and mental disability (Larroque et al., 2008; Saigal and Doyle, 2008). The etiology of Encephalopathy of Prematurity is multi-factorial with known reasons being: drugs, hypoxia & ischemia, hyperoxia and infection/inflammation. In this study, we focused on infection/inflammation.

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Current treatment aims at prevention, because a therapeutic approach to specifically tackle the pathophysiology of inflammation-induced developmental brain injury is not yet available (Favrais et al., 2014). Interestingly mesenchymal stem cells (MSCs) have been proposed to be beneficial in many inflammatory diseases, including developmental brain injury (Castillo-Melendez et al., 2013). MSCs secrete the anti-inflammatory protein TSG-6, which has been suggested to be responsible for a significant part of the beneficial effects of MSCs. For example, this was shown for models of myocardial infarction (Lee et al., 2009), acute lung injury (Danchuk et al., 2011) and skin wound healing (Qi et al., 2014).

Multiple functions of TSG-6 have been implied, including modulation of the extracellular matrix (Wisniewski et al., 1996; Baranova et al., 2011; Sanggaard et al., 2010), as well as direct cell interactions (Choi et al., 2011). Nevertheless, current knowledge about the physiological function of TSG-6 is still limited. It has been demonstrated to be essential during ovulation (Fülöp et al., 1997; Fülöp et al., 2003; Richards, 2005) and to be important in maintaining the physiological architecture of skin (Tan et al., 2011). Furthermore TSG-6 has strong anti-inflammatory properties, which have been intensively investigated in models of rheumatoid arthritis (Bárdos et al., 2001; Mindrescu et al., 2002). To date the role of TSG-6 in the developing brain remains unclear.

The aim of the current study was to evaluate 1) the developmental expression of TSG-6 in the neonatal rat brain, 2) the role of TSG-6 in perinatal inflammation with special focus on neuroinflammation, and 3) the effect of exogenous administration of TSG-6 on inflammation-induced brain injury using an established rat model (Prager et al., 2013; Brehmer et al., 2012).

2. Methods

All animal experiments were performed in accordance with international guidelines for good laboratory practice and institutional guidelines of the University Hospital Essen and were approved by the animal welfare committees of North Rhine Westphalia.

2.1. Animal treatment

Wistar rats, provided by the central animal facility of the University Hospital Essen, were used for all animal experiments. Developmental analysis was performed on healthy rats, decapitated on P1, P3, P5, P7, P9, P11, P13 or P15. Analysis of the impact of inflammation on neonatal brain development was performed on rats, treated on P3 with 0.25 mg/kg of LPS (Sigma-Aldrich) i.p. and decapitated on P3 + 0–24 h, P6 and P11. To investigate the pharmacological effects of recombinant human TSG-6 (rhTSG-6), rats were treated with four repetitive doses of 2.25 mg/kg rhTSG-6 every 12 h beginning on P3 (first dose 3 h prior to LPS injection) and decapitated on P3 + 6 h and P5.

2.2. Tissue collection

Animals received an overdose of chloral hydrate (200 mg of chloral hydrate per kg body weight in 0.1 ml injection volume per 10 g body weight) and were transcardially perfused with sterile PBS (except animals treated with LPS and decapitated on P3 + 0–24 h). Blood was collected from the right atrium before perfusion. Serum was prepared after clotting in uncoated tubes by centrifuging at 3000*g* for 5 min. Brain tissue was obtained by careful opening of the cranial cavity with subsequent brain extraction. Hemispheres or micro-dissected cortex, thalamus and striatum were isolated, snap-frozen in liquid nitrogen and stored at −80°C.

2.3. Western blotting

Protein from snap-frozen tissue was isolated by dissolving in RIPA-buffer (Sigma Life Science), containing PMFS (Sigma Life Science) and cOmplete Mini, Protease inhibitor cocktail (Roche). Cell compartments were centrifuged at 3000*g* for 10 min to separate the nuclei fraction and supernatant was further centrifuged at 17,000*g* for 20 min to separate the mitochondrial fraction. Cytosolic fraction was diluted in Laemmli buffer and denaturated at 95°C for 10 min. Proteins were separated by electrophoresis using 10–15% polyacrylamide gradient gels and transferred onto nitrocellulose membranes. Primary antibodies are listed below: TSG-6: goat-anti-hTSG-6 (1:80, R&D Systems, RRID: AB_2240677), cleaved caspase-3: rabbit-anti-caspase-3 (1:1000, Cell Signaling, RRID: AB_2070042), IBA1: rabbit-anti-IBA1 (1:1000, Wako Pure Chemical Industries). For quantitative analysis, HRP-linked secondary antibodies against the specific host species were used, visualized using Amersham ECL Plus (GE Healthcare) and detected by ChemiDoc XRS + System. Data were evaluated densitometrically using ImageLab software (Bio-Rad).

2.4. Multiplex ELISAs

Bioplex rat cytokine analysis was performed on serum and brain tissue. Data were acquired using Luminex 200 with IS2.3/xPONENT3.1 software and further processed with the R package nCal (Fong and Sebestyen, 2013). Standard curves were fitted by logistic regression (Ritz and Streibig, 2005) to estimate absolute concentration of analytes.

2.5. Real-time PCR

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies). Further processing included DNAse I reaction of 250–1000 ng RNA per batch and sample (Invitrogen, Life Technologies) and reverse transcription to cDNA using SuperScript II enzymes (Invitrogen, Life Technologies). The resulting cDNA was diluted using equivalent amounts of sterile distilled water. Relative quantification of cDNA amounts was performed using real-time PCR (StepOnePlus, Applied Biosystems) and the $2^{-\Delta\Delta C}{}_T$ (Livak and Schmittgen, 2001) method. The sequence of the TSG-6 forward primer was GTA GGA AGA TAC TGC GGT GAT GAA, the sequence of the TSG-6 reverse primer was GAC GGA CGC ATC ACT CAG AA and 6-FAM – TCC AGA AGA CAT CAT CAG CAC AGG AAA TGT – BHQ1 for the probe (BioTeZ).

2.6. Statistics

Statistical computing was done using R statistical environment (Team and R Core Team, 2012) and the dedicated integrated development environment RStudio. Additional packages used were gdata (Warnes, and with contributions from Ben Bolker, Gorjanc, G., Grothendieck, G., Korosec, A., Lumley, T., MacQueen, D., Magnusson, A., Rogers, J., Others, 2012), ggplot2 (Wickham, 2009), stringr (Wickham, 2012) and nCal (Fong and Sebestyen, 2013; Ritz and Streibig, 2005). Most data were $\log_2$ transformed to improve standard distribution of data. *p*-Values were calculated using analysis of variance models fitted into Tukey's Honest Significant Difference method as well as pairwise t-test using the Holm-Bonferroni method for multiple testing (#: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). Graphical presentation was generated using ggplot2.

3. Results

3.1. Developmental regulation of TSG-6 in the neonatal brain

We detected TSG-6 in the healthy neonatal rat brain at both mRNA and protein level by real-time PCR (Fig. 1) and Western blotting (data