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Neuregulin-1, the fetal endothelium, and brain damage in preterm newborns

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ABSTRACT

Objective: To assess the potential role for Neuregulin-1 (NRG1) as a systemic endogenous protector in the setting of perinatal inflammatory brain damage.
Methods: We measured *NRG1*-protein and mRNA levels in human umbilical venous endothelial cells (HUVECs) of different gestational ages at various durations of exposure to lipopolysaccharide (LPS). In parallel, we genotyped the donor individuals for SNP8NRG221533, a disease-related single nucleotide polymorphism in the 5′ region upstream of the *NRG1* sequence. Intracellular NRG1 localization was visualized by confocal microscopy. Furthermore we analyzed the relationship between SNP8NRG221533 genotype and neurodevelopmental outcome in children born preterm.
Results: We observed a positive dose–response-relationship between *NRG1*-mRNA and intracellular protein levels with both advancing gestational age and duration of LPS exposure in HUVECs. The presence of allele C at the SNP8NRG221533 locus was associated with an increased cellular production of NRG1 in HUVECs, and with a significantly reduced risk for cerebral palsy and developmental delay in children born preterm.
Interpretation: In conclusion, our data indicate that gestational age, duration of LPS exposure, and the SNP8NRG221533 genotype affect NRG1 levels. Our results support the hypothesis that NRG1 may qualify as an endogenous protector during fetal development.

1. Introduction

Preterm birth is associated with an increased risk of neonatal brain damage (Ferriero, 2004) and long-term developmental delay (Stephens and Vohr, 2009). Intrauterine infection leads to inflammatory responses (Dudley, 1997; Dammann et al., 2004; Romero et al., 2006, 2007) that contribute to prematurity and neonatal morbidity. The umbilical vessel endothelium is one of the first fetal interfaces exposed to inflammatory challenges, e.g. lipopolysaccharides (LPS). Indeed, endothelial cells have a remarkable capacity to respond to stressors (Dauphinee and Karsan, 2006; Magder et al., 2006).

Neuregulins (NRGs) play important roles during fetal brain (Bernstein et al., 2006), heart (Gassmann et al., 1995) and lung development (Dammann et al., 2003), and are involved in inflammatory processes (Xu et al., 2004, 2005). Several NRG isoforms (Meyer et al., 1997) are produced by alternative splicing (Wen et al., 1994; Falls, 2003). One of these, NRG1, appears to help signal the onset of surfactant synthesis in the fetal lung (Dammann et al., 2003) and might qualify as a brain protector in experimental ischemia (Xu et al., 2004, 2005). We have recently suggested that NRG1 might play a role not only in adult (Deadwyler et al., 2000; Gerecke et al., 2004), but also in neonatal brain disorders (Dammann et al., 2007).

The role of single nucleotide polymorphisms (SNPs) in inflammation-associated genes, e.g. interleukin (IL)-10, as risk-modulators for neonatal disorders has previously been investigated in a pilot study (Dordelmann et al., 2006; Dammann et al., 2009). Now we wanted to expand this scenario to include *NRG1* and one of its SNPs (SNP8NRG221533) that has been associated with schizophrenia (Stefansson et al., 2002, 2003).

Abbreviations: BPD, bronchopulmonary dysplasia; CAM, chorioamnionitis; CI, confidence interval; CLD, chronic lung disease; CP, cerebral palsy; cPVL, cystic periventricular leukomalacia; DD, developmental delay; NRG, Neuregulin; OR, odds ratio; PTL, preterm labor; PVE, periventricular echodensity; ROM, rupture of membranes; SGA, small for gestational age.

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We hypothesized, that

1. NRG1 is released by HUVECs in response to LPS-exposure;
2. *NRG1* gene expression and NRG1 protein production differ with both gestational age and LPS-treatment duration;
3. SNP8NRG221533 alters *NRG1* gene expression; and
4. there is a reduced risk for neonatal brain damage and developmental delay among children with a high producer allele of SNP8NRG221533.

2. Methods and patients

2.1. Cell culture

Umbilical cords were collected from babies born either before 30 weeks gestation (immature group) or after completion of 37th week of gestation (mature group). To reduce the likelihood of obtaining biased results all umbilical cords showing histological evidence for inflammation (funisitis) were excluded from our study. Endothelial cells were harvested from human umbilical cord vessels within 1–5 days of delivery (Bueter et al., 2006). Briefly, umbilical veins and arteries were cannulated with blunt needles, rinsed with sterile PBS buffer, and treated with type I collagenase (0.04%, GIBCO, Invitrogen, Karlsruhe) for 25 min at 37 °C. Cell-collagenase suspension was collected and centrifuged at 250*g* for 5 min at 4 °C. Cells were resuspended, and plated in endothelial cell growth medium containing 10% fetal calf serum (FCS). Cells were washed after 2–3 h of adherence and grown until confluence. We used cells up to the 5th passage for the experimental assays. Before treatment, cells were starved for two hours in Dulbecco's Modified Eagle's Medium (DMEM) containing 0.1% FCS. Treatment was performed with 100 ng/ml LPS for 1.5, 3, 6, 12, and 24 h. Untreated controls were kept in culture in DMEM for the same time.

2.2. NRG1 localization by confocal microscopy

HUVECs were grown on glass slides for 24 h and starved in serum-free DMEM for 2 h. Cells were either treated with LPS (100 ng/ml) for 30 min, 24 h or left in DMEM as a control for the same time at 37 °C. Immunofluorescence staining was performed as previously described (Padmakumar et al., 2004). Briefly, cells were fixed with 3% paraformaldehyde for 20 min followed by permeabilisation with 0.2% Triton X-100 for 2 min. After 1 h blocking in 10% normal goat serum, fixed cells were incubated with the specific primary NRG1 antibody (rabbit polyclonal IgG Neuregulin Ab-2; Neo Markers, Fremont, CA) at room temperature for 1–2 h. Cells were washed with PBS and incubated with the appropriate secondary antibody conjugated with Alexa568. Subsequently cells were incubated in 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, Heidelberg, Germany) for 10 min. Cells were mounted in Gelvatol/DABCO and analyzed using a Leica TCS–SP2 confocal laser scanning microscope.

2.3. Genotyping for SNP8NRG221533

We genotyped both HUVECs obtained from umbilical cords and lymphocyte DNA obtained from whole blood samples (Dordelmann et al., 2006). Genomic DNA was isolated using standard phenol–chloroform extraction. Polymerase chain reactions (PCR) were performed on 100 ng of genomic DNA using primers 5′-ACCT AAGATGTCCAAGAGACAG-3′ (forward) and 5′-GACTGGAAGCCAT GTATCTTTATTGT-3′ (reverse) and HotStart *Taq* DNA polymerase (5 U/μl, Qiagen, Hilden, Germany). Thirty-six cycles were performed with 15 min denaturation at 95 °C, 1 min annealing at 62° and 1 min extension at 72 °C. The artificially introduced mismatch in the reverse primer allowed for a subsequent allelic discrimination by the restriction enzyme *Rsa*I. PCR products were incubated with *Rsa*I (New England BioLabs, Frankfurt, Germany) at 37 °C overnight and restriction fragment length analysis (RFLP) was performed by 3% agarose gel electrophoresis.

2.4. ELISA

All sample-measurements were carried out in quadruplicates. Cells were scraped, lysed by sonication (3×30 s), lyophilized, and resuspended in 200 μl PBS. The amount of total protein was measured by DC Protein Assay (Bio-Rad, Munich, Germany). ELISA was performed using the Human NRG1-beta 1/HRG1-beta 1 Duo-Set (DY 377 R&D Systems, Wiesbaden, Germany). 96-well-plates were coated overnight with 100 μl capture antibody (4 μg/ml Heregulin Ab-1 Clone 7D5, Neo Markers, Fremont, CA) at 4 °C. After overnight blocking with 1% protease-free BSA and 5% sucrose, 100 μl of sample or standard were incubated overnight at 4 °C. 100 μl detection antibody (200 ng/ml Heregulin/Neuregulin Ab-2, biotin-labeled, Neo Markers), solved in PBS with 1% protease-free BSA and 2% normal goat serum was added for two hours. Cells were incubated in the dark in 50 μl Streptavidin HRP for 20 min followed by 100 μl substrate solution for 20 min. Substrate reaction was stopped by adding 50 μl 2 N H_2SO_4. Photometric extinction was measured at 450 vs. 570 nm. NRG1 concentration was calculated based on 1 mg of total protein and presented as% of controls.

2.5. Real-time RT-PCR

Total RNA was isolated using a guanidinium–phenol based extraction procedure (Abgene, Hamburg, Germany). One microgram of total RNA was subjected to reverse transcription using a First-Strand cDNA Synthesis Kit (GE Healthcare, Munich, Germany). Real-time PCR was performed using a commercially available primer assay for *NRG1* and a SYBR Green Master Mix (Qiagen, Hilden, Germany) following the manufacturer's protocol with 40 cycles and an annealing at 60 °C on a Sequence Detection System 7000 (Applied Biosystems). β-Actin served as the housekeeping gene and was amplified on the same plate using primers 5′-AGATGACCCAGATCATGTTTGAG-3′ and 5′-GAGTCCATCACGATG CCAGTG-3′. We calculated means and DCt values using the ABI PRISM 7000 SDS software (Applied Biosystems, Darmstadt, Germany) for relative quantification. Corresponding control values were subtracted from treated samples to calculate DDCt values. A negative result indicates a higher *NRG1* gene expression in the treated sample compared to the respective untreated control.

2.6. Statistics

p-Values were calculated using the two-tailed *t*-test for equal variances. Equality of variances was tested by Levene Test. Potential associations between genotypes, antenatal characteristics, and clinical outcome were calculated using logistic regression and the one-sided Cochran–Armitage test for trend (publicly available syntax was obtained from http://listserv.uga.edu/). All analyses were performed with SPSS 15.0 (SPSS Inc., 2006).

2.7. Study population

We evaluated 54 children <32 weeks gestation from the Developmental Follow Up Program at Hannover Medical School (Hannover, Germany). Children were at least two years of age. A comprehensive physical examination and the Denver developmental screening (DDST) II test were administered by one pediatrition. Clinical data were abstracted from medical charts. Peripheral