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Strain influences on inflammatory pathway activation, cell infiltration and complement cascade after traumatic brain injury in the rat

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ABSTRACT

Increasing evidence suggests that genetic background affects outcome of traumatic brain injuries (TBI). Still, there is limited detailed knowledge on what pathways/processes are affected by genetic heterogeneity. The inbred rat strains DA and PVG differ in neuronal survival following TBI. We here carried out global expressional profiling to identify differentially regulated pathways governing the response to an experimental controlled brain contusion injury. One of the most differentially regulated molecular networks concerned immune cell trafficking. Subsequent characterization of the involved cells using flow cytometry demonstrated greater infiltration of neutrophils and monocytes, as well as a higher degree of microglia activation in DA compared to PVG rats. In addition, DA rats displayed a higher number of NK cells and a higher ratio of CD161bright compared to CD161dim NK cells. Local expression of complement pathway molecules such as C1 and C3 was higher in DA and both the key complement component C3 and membrane-attack complex (MAC) could be demonstrated on axons and nerve cells. A stronger activation of the complement system in DA was associated with higher cerebrospinal fluid levels of neurofilament-light, a biomarker for nerve/axonal injury. In summary, we demonstrate substantial differences between DA and PVG rats in activation of inflammatory pathways; in particular, immune cell influx and complement activation associated with neuronal/axonal injury after TBI. These findings suggest genetic influences acting on inflammatory activation to be of importance in TBI and motivate further efforts using experimental forward genetics to identify genes/pathways that affect outcome.

1. Introduction

Traumatic brain injury (TBI) is one of the leading factors of neurological disability and death among young individuals worldwide. Trauma to the brain results in a primary mechanical damage characterized by disruption of the brain parenchyma and blood vessels, which initiates a complex cascade of molecular events and physiological processes. Some of these processes that take place hours and days after the primary insult may lead to further injury on nerve cells and axons, i.e. secondary brain damage. Neurological disability and clinical outcome depends on the combined extent of both acute and subacute injury processes. This provides a potential therapeutic window for efforts directed at reducing secondary neuronal damage.

In the last decades, many studies have explored the role of the immune system in TBI and it is now recognized that inflammation plays a major role in secondary injury, not only immediately after the trauma, but perhaps even after months and years (Ramlackhansingh et al., 2011). The initial impact leads to immediate cell death, which together with disruption of blood brain barrier (BBB) integrity leads to an inflammatory reaction. This reaction is characterized by activation of local glial cells, with up-regulation and release of inflammatory molecules such as complement factors, chemokines and cytokines, and infiltration of blood derived immune cells into the brain tissue (Kumar and Loane, 2012; Shlosberg et al., 2010). However, the inflammatory response is complex and both detrimental and protective aspects of diverse pathways and molecules have been described in previous studies (Morganti-Kossmann et al., 2002, 2007).

Studies in human diseases such as multiple sclerosis and rheumatoid arthritis, where the immune system plays an instrumental role, has generated the term "complex diseases" in which disease risk is dependent on a complex interplay between environmental and genetic factors (Kim and Moudgil, 2009; McElroy and Oksenberg, 2011). Not surprisingly most of the genetic polymorphisms regulating disease susceptibility identified so far affect immune related molecules and pathways. The identification of

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these polymorphisms has been achieved by association studies in humans aided by the use of inbred strains and forward genetics in animal disease models (Marta et al., 2010; McElroy and Oksenberg, 2011). Although comparative strain differences have been used for years to elucidate the mechanisms regulating susceptibility to autoimmune conditions such as EAE, so far only limited efforts have been made to test if this approach is feasible also in primarily non-autoimmune disease models, such as traumatic nerve injuries (Kornblum, 1968). We have found considerable rat strain differences in the inflammatory response and outcome in previous studies on nerve, spinal cord and brain trauma, suggesting that genetic factors acting on the inflammatory response are important for outcome (Al Nimer et al., 2011; Bellander et al., 2010; Birdsall Abrams et al., 2007; Dominguez et al., 2008; Lidman et al., 2003).

In recent years there is an increasing awareness that genetic factors that vary between individuals may affect outcome of TBI (Cederberg and Siesjo, 2010; Jordan, 2007; Reid et al., 2010; Sun and Jiang, 2008; Zhou et al., 2008). Genetic variability may influence pathophysiological processes triggered by TBI, in turn of relevance for outcome and possibly also response to treatment (Dardiotis et al., 2010; Maas et al., 2010; McAllister, 2010; Zhou et al., 2008). DA (RT1^{av1}) and PVG-RT1^{av1} are two inbred rat strains that share MHC haplotypes, but differ substantially in neuronal survival following TBI and ventral root avulsion (Al Nimer et al., 2012; Strom et al., 2012). We here hypothesized that genetic heterogeneity would affect the pathophysiological mechanisms and nerve cell death in a standardized brain contusion model and that it would be possible to determine quantitative and qualitative differences between DA (RT1^{av1}) and PVG-RT1^{av1}.

Using global expression profiling we found several differentially regulated functional networks, out of which the strongest were related to inflammation, such as inflammatory cell influx and complement activation. Further characterization of the immune cell influx and local cell activation demonstrated that the DA strain displayed greater influx of neutrophils, monocytes/macrophages, and NK cells, as well as more intense microglia activation and higher local expression of complement factors such as *C1*, *C3* and complement receptor 2 (*CR2*). Higher levels of neurofilament-light in cerebrospinal fluid (CSF) suggested that increased inflammatory activation in the DA strain was associated with increased secondary nerve injury. The results strengthen the notion that genetic heterogeneity affects inflammatory regulation in primarily non-autoimmune traumatic disease models and should be further explored in order to understand the pathophysiology of TBI.

2. Methods

2.1. Animals and surgery

The DA (RT1^{av1}) strain was originally provided by Professor Hans Hedrich (Medizinische Hochschule, Hannover, Germany), while the MHC congenic PVG-RT1^{av1} (hereafter called PVG) strain was obtained from Harlan UK Ltd. (Blackthorn, UK). All animals were bred in our in-house breeding facility with 12 h light/dark cycles and fed standard rodent chow and water *ad libitum*. Experimental traumatic brain contusion was performed in male animals weighing approximately 230–300 g, at an age of 10–14 weeks, under deep isoflurane anesthesia using the weight drop injury model, modified by Feeney, as described previously (Al Nimer et al., 2011; Feeney et al., 1981). In brief, the rats were placed in a stereotaxic frame and after skin incision the skull bone was removed 3 mm behind and 2.3 mm lateral to the bregma. A brain contusion was performed by the use of a weight drop device and the piston was allowed to compress the brain at a maximum depth of 3 mm. The animals were euthanized by deep isoflurane anesthesia followed by CO_2 and perfused with PBS at different time points after the injury. All experiments were approved by the local ethical committee for animal experimentation.

2.2. Array hybridization and qRT–PCR

The brain was extracted and a sample corer (Fine Science Tools, Heidelberg, Germany) was used to collect a $5 \times 5 \times 6$ mm piece of tissue consisting of the contusion core, the pericontusional cortex, the underlying hippocampus and part of the thalamus. Preparation of mRNA was done as previously reported (Al Nimer et al., 2011).

The microarray analysis was performed at the microarray core facility of Karolinska Institutet using Affymetrix Rat gene 1.0 ST Array chips (Affymetrix, Santa Clara, CA) as described before (Strom et al., 2012). The microarray data are available in MIAME-compliant (minimal information about a microarray experiments) format at the ArrayExpress Database (http://www.ebi.ac.uk/arrayexpress) under accession code E-MTAB-795. Preparation of cDNA and qRT–PCR was done as described before (Al Nimer et al., 2011). *Gapdh* and *Hprt* were used as housekeeping genes. The sequences of the used primers are shown in Table 1.

2.3. Microarray analyses

Statistical analyses of microarray data were done in Partek Express®. Copyright, Partek Inc. Partek and all other Partek Inc. product or service names are registered trademarks or trademarks of Partek Inc., St. Louis, MO, USA. The expression data were imported into the software Partek Express where they are normalized using the RMA algorithm (Irizarry et al., 2003). Mixed model analysis of variance was used to obtain a *p* and a fold change value and to examine the significance and degree of the differences between DA and PVG for every expressed target. The data were then uploaded in Ingenuity Pathway Analysis software (IPA 9.0, http://www.ingenuity.com), a web-based bioinformatics tool, in order to identify functional networks and pathways. Transcripts with a predetermined *p* value and fold change were up-loaded for functional analysis of networks and biological pathway analysis with IPA.

Based on the selected genes (focus genes) a functional analysis of the networks was performed in IPA in order to identify the biological functions and/or diseases that were most significant to the molecules in each network. IPA calculates also a significance score for each biological function in a network. The score is generated using a *p*-value calculation and is displayed as the negative log of that *p*-value. This score indicates the likelihood that the assembly of a set of focus genes in a network could be explained by random chance alone. A score of 2 indicates that there is a 1 in 100 chance that the focus genes are together in a network due to random chance.

Canonical pathways analysis identified the pathways from the IPA library of pathways that were most significant to the data set. Molecules from the data set that met the *p* value and fold change cutoff and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways: (1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway and (2) Fisher's exact test was used to calculate a *p*-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

The uploaded data were also used to generate a pathway graphical representation for the canonical pathway with the highest