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Infection of human airway epithelial cells by different subtypes of Dobrava-Belgrade virus reveals gene expression patterns corresponding to their virulence potential

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ABSTRACT

Dobrava-Belgrade virus (DOBV) is a pathogen causing hemorrhagic fever with renal syndrome in Europe. Virulence and case fatality rate are associated with virus genotype; however the reasons for these differences are not well understood. In this work we present virus-specific effects on the gene expression profiles of human lung epithelial cells (A549) infected with different genotypes of DOBV (Dobrava, Kurkino, and Sochi), as well as the low-virulent Tula virus (TULV). The data was collected by whole-genome gene expression microarrays and confirmed by quantitative real-time PCR. Despite their close genetic relationship, the expression profiles induced by infection with different hantaviruses are significantly varying. Major differences were observed in regulation of immune response genes, which were especially induced by highly virulent DOBV genotypes Dobrava and Sochi in contrast to less virulent DOBV-Kurkino and TULV. This work gives first insights into the differences of virus - host interactions of DOBV on genotype level.

1. Introduction

Hantaviruses are the only members of the family *Bunyaviridae* to be harbored by small mammals. They cause human zoonoses known as hemorrhagic fever with renal syndrome (HFRS) in the Old World and hantavirus cardiopulmonary syndrome (HCPS) in the New World (Krüger et al., 2015a). In Europe the rodent-borne *Puumala virus* (PUUV) carried by *Myodes glareolus* (Brummer-Korvenkontio et al., 1980; Niklasson and LeDuc, 1984) and *Dobrava-Belgrade virus* (DOBV) carried by different *Apodemus* species (Avsic-Zupanc et al., 1992; Klempa et al., 2005, 2008) are the main causative agents of hantavirus-associated disease (Vaheri et al., 2013). While PUUV causes mainly a mild form of HFRS also known as nephropathia epidemica (Makary et al., 2010), DOBV is responsible for human infections of varying severity according to the specific DOBV genotype (Dzagurova et al., 2012; Hofmann et al., 2014; Klempa et al., 2013). *Tula virus* (TULV) carried by *Microtus* voles was shown to infect humans in Europe, but rarely causes disease without any fatalities known so far. Symptomatic patients with verified TULV infection show typical HFRS progression, like fever, headache, thrombocytopenia, oliguria, and elevated levels of creatinine, however the low number of known cases ($n=3$) does not allow a statistical validation (Klempa et al., 2003a; Mertens et al., 2011; Reynes et al., 2015; Zelená et al., 2013).

For many years DOBV was in focus of scientific debates about virus species demarcation as variants of the virus were found in different species of *Apodemus* mice, namely *Apodemus agrarius*, *Apodemus flavicollis*, and *Apodemus ponticus*, even co-existing within the same natural focus with occurring spill-over infections between the designated hosts (Klempa et al., 2003b; Schlegel et al., 2009). Since 2012 there is a consensus within the international hantavirus community to define genetic variants of DOBV as genotypes named accordingly to the spot of their first detection (Klempa et al., 2013). The so far known DOBV genotypes are Dobrava (from *A. flavicollis* [earlier described as DOBV-Af]), Kurkino (from *A. agrarius* [DOBV-Aa]), and Sochi (from *A. ponticus* [DOBV-Ap]).

Alongside with differences in host preference, genetic information, and geographic occurrence, DOBV genotypes have a different probability to cause HFRS with severe course of infection. While DOBV-Kurkino tends to cause a milder form of hantavirus disease with a case fatality rate of 0.5–0.9%, DOBV-Dobrava, and DOBV-Sochi induce severe HFRS more frequently and lead to

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fatality rates of more than 10% (with up to 14.5% for DOBV-Sochi). The classification criteria of clinical severity of DOBV infections are better defined than for TULV and differentiate between mild, moderate, or severe. The typical symptoms used for severity classification are: fever duration, systolic blood pressure level, intensity of hemorrhagic syndrome, extent and duration of oliguria, elevation of serum creatinine, proteinuria, and in severe cases anuria, edema, and kidney rupture. (Klempa et al., 2008, 2013; Krüger et al., 2015b; Leshchinskaia et al., 1990).

While being pathogenic to humans, hantaviruses usually do not cause disease in their natural host, being virulent only in spill-over infections (Hardcastle et al., 2016; Popugaeva et al., 2012; Safronetz et al., 2012) or in mice (*Mus musculus*) provided with a humanized immune system (Kobak et al., 2015). Many speculations have been made about the reason for the divergent virulence potential of DOBV genotypes, including receptor preference towards different beta-integrin molecules, as well as the varying potential to cope with the human innate immune response after infection (Krautkrämer and Zeier, 2008; Popugaeva et al., 2012; Rang, 2010; Schönrich et al., 2008). As immunopathogenesis in response to hantavirus infection contributes to HFRS (Schönrich et al., 2015; Krautkrämer and Zeier, 2014; Rang, 2010), the modulation of innate immune response is a crucial point in investigating DOBV pathogenesis.

Infections with pathogenic hantaviruses are shown to modulate barrier function of endothelial cells and function of blood platelets by recruitment of ß3-integrins (Gavrilovskaya et al., 1998). Increased levels of inflammatory cytokines were shown to occur in patients but also in infected cell cultures (Geimonen et al., 2002; Mackow and Gavrilovskaya, 2009; Schönrich et al., 2008). These increased inflammatory responses contribute to the generation of immune pathogenesis and so called cytokine storm during HFRS. Specific interferon stimulated genes (ISG) like the antiviral proteins MX1 (Haller and Kochs, 2011) or RIG-I (Imaizumi et al., 2005) have been previously used to monitor the antiviral response of the innate immune system of infected cells (Matthys and Mackow, 2012). However, a heuristic comparison of the innate immune system response to infection by DOBV and its different genotypes has never been shown.

To elucidate possible reasons for the varying HFRS severity of different DOBV genotypes we performed a study to track the impact of DOBV infection on the gene expression profiles of human lung epithelial cells (A549). Lung tissue cells, as a first line of defense after inhalation of hantavirus particles, decide about the further progression of disease. Moreover, A549 cells have been widely used as an *in vitro* model system for hantavirus infections including gene expression profiling (Lee et al., 2011; Oelschlegel et al., 2007; Popugaeva et al., 2012; Shim et al., 2011; Stoltz and Klingström, 2010). Our study was conducted by an analysis of the cellular gene expression profiles via whole-genome gene expression microarray and quantitative real-time PCR (qPCR) techniques.

2. Materials and methods

2.1. Virus cultivation

DOBV [genotypes Dobrava Slo 97110 (Avsic-Zupanc et al., 1992), Kurkino GER/08/131/Af (Popugaeva et al., 2012), and Sochi Ap/Sochi/hu (Dzagurova et al., 2012)] and TULV [Moravia Ve6P3951129 (Vapalahti et al., 1996)] were grown on Vero E6 cells (ATCC CRL 1586) in T175 cell culture flasks under standard cell culture conditions (DMEM medium, 5% FCS, 37 °C, 0.005 multiplicity of infection [MOI]). 7 d post infection (p.i.) cell disruption by freeze/thaw cycles and subsequent sucrose cushion ultracentrifugation was performed to receive an enriched virus preparation. Viral stocks were titrated by focus forming unit test and stored in aliquots at −80 °C for further use. Biosafety level 3 facilities were used for virus cultivation and experimental infections.

2.2. Infection and sampling of lung epithelial cells

A549 human lung carcinoma epithelial cells (DSMZ ACC 107) were cultured under standard conditions in 12-well cell culture plates with and without microscopy glass slides. Cells were infected with the described viruses at MOI of 5 and the virus was adsorbed for 1 h. Mock infections were performed using culture medium free of virus. After 12 h samples for total RNA extraction were taken by usage of TRIzol reagent (Life Technologies) according to manufacturer's specifications. At the same time point samples were taken for Immunofluorescence assay (IFA) as infection control by fixating glass slides containing infected cells with 4% methanol-free paraformaldehyde. Additional RNA samples for qPCR quantification were taken at 1 h, 2 h, 4 h, 6 h, 10 h, and 12 h by usage of RLT buffer and the appendant RNeasy Mini Kit (Qiagen).

2.3. RNA labeling and hybridization

Sample labeling was performed as detailed in the Agilent "One-Color Microarray-Based Gene Expression Analysis" protocol (version 5.7, part number G4140-90040). For the linear T7-based amplification step, 100 ng of each total RNA sample was used. To produce Cy3-labeled cRNA, the RNA samples were amplified and labeled using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies) following the manufacturer's protocol. Yields of cRNA and the dye-incorporation rate were measured with the ND-1000 Spectrophotometer (NanoDrop Technologies). The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). Briefly, 600 ng Cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight (17 h, 65 °C) to Agilent Whole Human Genome Oligo Microarrays 8×60 K (including 27,985 individual Entrez gene RNAs) using Agilent's recommended hybridization chamber and oven. Finally, the microarrays were washed once with the Agilent Gene Expression Wash Buffer 1 for 1 min at room temperature, followed by a second wash with preheated Agilent Gene Expression Wash Buffer 2 (37 °C) for 1 min. The last washing step was performed with acetonitrile. Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies).

2.4. Data analysis

The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. For determination of differential gene expression FES-derived output data files were further analyzed using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware). Ratios were calculated by dividing sample signal intensity through control signal intensity. The signal intensities from the single-experiment raw data lists were normalized by dividing the intensity values by their median. Putative candidate genes were selected based on a minimum fold change (FC) ≥ 2 and p-value ≤ 0.01. The calculation of merged ratios for replicate experiments was performed by calculating pairwise log-ratios and log-ratio error and the subsequent combination to one ratio in an error-weighted averaging procedure. Data visualization was done via generation of heat maps from the