Molecular Mechanisms of West Nile Virus Pathogenesis in Brain Cells

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We analyzed the response of human glioma cells to West Nile virus infection by investigating host transcriptional changes. Changes in expression of 23 genes showed similarities to those in other neurodegenerative diseases. These changes may be useful as potential biomarkers and elucidate novel mechanisms behind the neuropathology of infection with this virus.

West Nile virus (WNV), a member of the family Flaviviridae, is the etiologic agent of West Nile fever. Since WNV is neurotropic, severe human meningoencephalitis is a common complication of infection and results in a considerable number of deaths. The medulla of the brainstem in the central nervous system (CNS) is the primary target of WNV (1).

WNV replicates in a wide variety of cell types, and studies have traditionally been carried out in Vero (green monkey kidney) and C6 (mosquito) cells. However, little work has been done with CNS cells. We conducted a global transcriptional analysis of human glioblastoma cell response to infection with WNV during peak virus production to determine the crucial virus-host interactions that take place during a severe neuroinvasive attack and identify putative mechanisms involved in WNV pathogenesis. The factors governing the development of neurologic disease, host immune response, patterns of clinical features, and outcomes are poorly understood in those infected with neurotropic flaviviruses (2).

A total of 173 genes were differentially expressed, many of which were not found in previous transcriptional studies of other flaviviruses (3). From these, 23 genes were identified that may play a role in cellular neurodegeneration. These novel changes induced by WNV may serve as biomarkers and help explain the neuropathologic features observed.

The Study

Most laboratory studies of WNV infections have been carried out in animal cell lines or human cell lines of non-CNS origins. In this study, human glioblastoma (A172) cells were found to be a useful laboratory model for investigating WNV infections. A172 (human glioblastoma) cells were maintained at 37°C in Dulbecco modified Eagle medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum. Confluent monolayers of A172 cells were infected with the Sarafend strain of WNV at a multiplicity of infection of 1. Twenty-four hours after infection, cells showed signs of cytopathic effects (cell-rounding) and produced high virus titer (10^8 PFU/mL). This demonstrated the highly susceptible nature of the neuralgial cells to WNV infection. Batches of cells were infected for microarray experiments, and a quantitative polymerase chain reaction was used to verify the reproducibility of the changes in gene expression.

Total cellular RNA was extracted from mock-infected and infected cells by using the RNeasy Mini kit and QIAshredder (Qiagen, Hilden, Germany). The CyScribe first-strand cDNA labeling kit (Amersham Biosciences, Piscataway, NJ, USA) was used to incorporate fluorescent Cy3-dCTP or Cy5-dCTP (Amersham Biosciences) into cDNA probes. The probes were subsequently purified by using CyScribe GFX purification columns (Amersham Biosciences). Equal amounts of labeled cDNA probes (=25 pmol) were combined for microarray hybridizations. Human 1A microarrays (Agilent Technologies, Palo Alto, CA, USA) were used, and hybridizations were performed on a Lucidea SlidePro Hybridizer (Amersham Biosciences). The microarray experiment was carried out in triplicate: 1 of the microarrays was with a dye-swap labeling to prevent skew in the results due to bias in CyDye incorporation.

Analyses of the scanned microarray images were performed with BRB ArrayTools version 3.1 (developed by R. Simon and A.P. Lam, National Cancer Institute, Bethesda, MD, USA, and available at http://linus.nci.nih.gov/BRB-ArrayTools.html), and normalized by using the Lowess method. A stringent lower limit threshold was set at 3 standard deviations of the pixel intensities of the negative control spots, and images were screened for changes in expression values of at least 2-fold. The differentially regulated genes were separately uploaded into EASE (4) to determine the biologic themes that were significantly overrepresented (Fisher exact test with p values < 0.01). A total of 173 cellular genes were identified by ArrayTools to be differentially expressed in the WNV-infected A172 cells. EASE clustered 39 of the upregulated genes and 41 of the downregulated genes into specific functional groups (available at http://sps.nus.edu.sg/~kohweele/awn_genes.htm).

Functional classes that were found to be enriched in the upregulated genes encompassed those related to immunity, responses to external stimulus and pathogens, and apoptosis. Genes relating to the ubiquitin cycle, transcription...
regulation, and other physiologic processes were also identified by EASE. Functional classes that were downregulated were not commonly observed in a virus infection system. For instance, genes relating to the mitochondria, ribosomes, and protein biosynthesis were highly overrepresented in down regulation (available at http://sps.nus.edu.sg/~kohweele/awn_genes.htm). From this set of genes, a group of 23 genes that may provide the molecular basis for the observed pathogenesis in the A172 cells was identified (Table 1).

A quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was carried out to ensure an independent assessment of the microarray results. Genes for the qRT-PCR were selected to represent the broad spectrum of identified functional classes from the microarrays. The hypoxanthine guanine phosphoribosyltransferase gene was used as an internal control (primers for the PCR can be found at http://sps.nus.edu.sg/~kohweele/awn_genes.htm). RNA was reverse-transcribed by using SuperScript III (Invitrogen, Carlsbad, CA, USA), and a real-time PCR was carried out with Platinum SYBR Green (Invitrogen). A negative template control that contained all SYBR green reagents except DNA was performed in parallel on the iCycler iQ (Bio-Rad Laboratories, Hercules, CA, USA). The results corroborated the microarray data, thereby verifying the accuracy of the statistical analysis (Table 2). However, the qRT-PCR showed greater dynamism in fold changes than the microarray results because of the greater sensitivity of PCR compared with fluorescent detection.

### Conclusions

In this study, WNV infection of human brain glioma cells showed advanced cytopathic effects within 24 h after infection and produced high virus yields. This demonstrated that human glioma cells from CNS are susceptible to WNV infection and are suitable for the study of viral pathogenesis.

The activation of the innate antiviral immune response pathways is often the primary cause of pathologic effects. The presence of double-stranded RNA replication complexes from viral origins causes the transcriptional activation of the interferon-α/β (IFN-α/β) or type-I IFN pathways (5). In this study on glioma cells, the activation of numerous interferon-induced proteins (such as IFIT1,

| Table 1. Differentially regulated genes involved in pathogenesis of A172 cells infected with West Nile virus |
|---------------------------------------------------------|
| **Gene** | **Gene name** | **Fold change** |
|-----------------------------|------------------|------------------|
| **Immune response related** | | |
| OAS3 | 2'-5'-oligoadenylate synthetase 3, 100 kDa | 2.32 |
| OASL | 2'-5'-oligoadenylate synthetase-like | 3.46 |
| FIT1 | Interferon-induced protein with tetracopeptide repeats 1 | 10.74 |
| IFIT2 | Interferon-induced protein with tetracopeptide repeats 2 | 3.76 |
| IFI27 | Interferon, α-inducible protein 27 | 4.03 |
| IFITM1 | Interferon-induced transmembrane protein 1 (9-27) | 12.00 |
| IFITM2 | Interferon-induced transmembrane protein 2 (1-80) | 3.04 |
| G1P2 | Interferon, α-inducible protein (clone IFI-15K) | 9.50 |
| HLA-C | Major histocompatibility complex, class I, C | 2.20 |
| INDO | Indoleamine-pyrorole 2,3 dioxygenase | 3.38 |
| PTX3 | Pentaxin-related gene, rapidly induced by interleukin-1β | 3.44 |
| **Apoptosis related** | | |
| TNFSF14 | Tumor necrosis factor (TNF) (ligand) superfamily, member 14 | 2.19 |
| NFKBIA | Nuclear factor of kappa light polypeptide gene enhancer | 4.13 |
| TRAF1 | TNF receptor-associated factor 1 | 2.01 |
| SAT | Spermidine/spermine N1-acetyltransferase | 2.18 |
| **Mitochondria related** | | |
| SDHC | Succinate dehydrogenase complex, subunit C | −2.31 |
| COX5B | Cytochrome c oxidase subunit Vb | −2.13 |
| COX6B | Cytochrome c oxidase subunit Vb | −2.41 |
| ATP5G1 | ATP synthase, mitochondrial F0 complex, subunit c, isoform 1 | −2.64 |
| ATP5C1 | ATP synthase, mitochondrial F1 complex, γ polypeptide 1 | −3.82 |
| ATP5J | ATP synthase, mitochondrial F0 complex, subunit F6 | −2.11 |
| ATP5B | ATP synthase, mitochondrial F1 complex, β polypeptide | −2.17 |
| ATP5A1 | ATP synthase, mitochondrial F1 complex, α subunit, isoform 1 | −2.21 |
| ATP5O | ATP synthase, mitochondrial F1 complex, O subunit | −2.00 |
| ATP5F1 | ATP synthase, mitochondrial F0 complex, subunit b, isoform 1 | −2.43 |
| PRDX5 | Peroxiredoxin 5 | −2.74 |
| PRDX3 | Peroxiredoxin 3 | −2.27 |
| **Protein biosynthesis related** | | |
| NACA | Nascent-polypeptide-associated complex polypeptide | −2.17 |
IFIT2, IFI27, IFITM1, IFITM2, and G1P2) lends support to this mechanism of pathogenicity. Glial cells are useful in this study because they are immune cells of CNS origin. Activated glial cells have macrophagic activity and are primed to respond to the virus, therefore allowing the display of immune-mediated neuropathologic changes that reflect conditions in the natural CNS host cells. Glial cells can also activate the type-II (IFN-\(\gamma\)) pathway and modulate the immune response by regulating cell trafficking of various leukocytes, including macrophage activation and stimulation of specific T cells responsible for cytotoxic immunity (6).

An example of this activation was finding that the HLA-C gene coding for the major histocompatibility complex class I (MHC-I) antigens was upregulated in the A172 cells. Peptides derived from endogenous intracellular proteins are generally bound by the MHC-I molecules for presentation, thus paving the way for cell cytotoxicity in cellular immunity. In mice, the targeted killing of WNV-infected cells by CD8+ T cells may result in the severe neurologic disease often observed in WNV infections (7).

In addition, indoleamine 2,3 dioxygenase (INDO) was observed to be upregulated in WNV-infected A172 cells. Increased production of INDO by glial cells causes neuronal injury in neuroinflammatory diseases (8). The upregulation of the pentaxin-related gene (PTX3) is also implicated in local tissue damage through the amplification of inflammation in innate immunity (9).

A group of genes causing apoptosis was also found to be upregulated, thus elucidating pathways linking virus replication to apoptosis. These genes include the tumor necrosis factor superfamily (TNFSF14), nuclear factor of \(\kappa\) light-chain gene (NFKBIA), TNF receptor-associated factor (TRAF1), and spermidine/spermine N1-acetyltransferase (SAT). This highly conserved process of cellular self-destruction serves to limit the spread of WNV (10).

A major group of genes relating to mitochondria was found to be downregulated. Mitochondrial defects due to respiratory-chain dysfunction and free-radical formation have been associated with neurodegenerative diseases such as Huntington disease, Parkinson disease, and Friedreich ataxia (11). Neurologic symptoms of these diseases were also observed in WNV-infected patients (12), suggesting similar neurodegenerative pathways.

The activity of genes belonging to the energy synthesis pathways was decreased. These genes included succinate dehydrogenase (SDHC), cytochrome c oxidase (COX5B/COX6B), and various genes of the ATP synthase complex (ATP5G1, ATP5C1, ATP5B, ATP5A1, ATP5O, and ATP5F1). Decreased energy production from the downregulation of these genes is known to cause severe neurodegeneration (13). Two antioxidant enzymes of the peroxiredoxin family (PRDX5 and PRDX3) were also downregulated. The increase in oxidative stress induced by reactive oxygen species can create a proinflammatory condition that results in CNS pathology and leads to Alzheimer disease and Down syndrome (14). Downregulation of the nascent polypeptide-associated complex (NACA) can also lead to similar neurodegeneration (15).

In summary, this global transcriptional study showed a complex network of WNV-induced A172 cell interactions during infection. The examination of glial A172 cell response has provided insights into the molecular mechanisms behind the observed neuronal pathology in WNV encephalitis.

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