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UPREGULATION OF INTERFERON-GAMMA-INDUCED GENES DURING PRION INFECTION

Laura R. Moody¹, Allen J. Herbst², and Judd M. Aiken²

¹Cellular and Molecular Biology Graduate Program; Department of Comparative Biosciences; University of Wisconsin, Madison, Wisconsin, USA

²Centre for Prions and Protein Folding Diseases, Edmonton, Alberta, Canada

Abstract

Global gene expression analysis allows for the identification of transcripts that are differentially regulated during a disease state. Many groups, including our own, have identified hundreds of genes differentially regulated in response to prion infection. Eleven transcripts, upregulated in the brains of prion-infected animals, which were classified in the literature as stimulated by the cytokine interferon-gamma (IFN- γ), were identified. This is intriguing, as IFN- γ has recently been detected in the brains of prion-infected animals. Quantitation of several genes, categorized as IFN- γ inducible, by quantitative real-time polymerase chain reaction (qRT-PCR) confirms that these transcripts are upregulated. Future approaches for delineating the role of IFN- γ -induced transcripts and their function in prion infection are described.

Prion diseases are infectious, neurodegenerative disorders that are ultimately fatal. These diseases include Creutzfeldt–Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and chronic wasting disease (CWD) in cervids. The infectious agent is the protease-resistant, misfolded form (PrP^{Sc}) of the cellular prion protein (PrP^C) (Prusiner, 1982), which accumulates and aggregates in most prion infections. Spongiform vacuolization of the central nervous system (CNS), proliferation of glial cells, and neuronal loss are pathological hallmarks of prion diseases (Collinge, 2001).

Subtractive hybridization of cDNA libraries and, more recently, microarray analysis allow for global identification of transcripts differentially expressed in response to various stimuli. Several groups, including our own, employed these techniques to identify gene expression changes in a variety of tissues in response to different prion strains throughout prion infection (Duguid et al., 1988, 1989; Duguid & Dinauer, 1990; Diedrich et al., 1991, 1993; Duguid & Trzepacz, 1993; Dandoy-Dron et al., 1998; Riemer et al., 2000, 2004; Baker & Manuelidis, 2003; Baker et al., 2004; Booth et al., 2004a, 2004b; Brown et al., 2004, 2005; Xiang et al., 2004, 2007; Skinner et al., 2006; Moody et al., 2009). A consistent feature of these studies is the overrepresentation of transcripts related to astrocytes and microglia, the cells proliferating in the brain during disease. Many of these transcripts are associated with the Gene Ontology (GO) term “immune response.” Prion diseases do not elicit a humoral

immune response (Porter et al., 1973); therefore, the transcripts related to the “immune response” GO term are likely related to microglia, the CNS-specific immune cells or immune system mediators produced by astrocytes (Gehrmann et al., 1995; Farina et al., 2007). Moody et al. (2009) analyzed this subset of our data looking for transcripts expressed through similar signaling pathways to gain a better understanding of the molecular signaling events that are occurring in the prion-infected brain. Unexpectedly, 11 transcripts upregulated were identified in both preclinical and clinical prion-infected brain tissue whose induction is postulated to be stimulated by interferon (IFN)- γ , a cytokine recently identified as upregulated in prion-infected brain (Tribouillard-Tanvier et al., 2009).

METHODS

Animals

C57Bl/6 mice were intraperitoneally (ip) inoculated with 10% brain homogenate (in phosphate-buffered saline [PBS]) prepared from a mouse clinically infected with the RML strain of prion agent. Control mice were mockinoculated with 10% brain homogenate prepared from uninfected C57Bl/6 mice. Mice were sacrificed at two preclinical timepoints (108 and 158 d post inoculation [dpi]) and at the appearance of clinical symptoms (198 dpi).

RNA Purification, Array Hybridization, and Data Analysis

Total RNA purification, preparation, and hybridization to Affymetrix Mouse Genome 430 2.0 arrays was previously described by Moody et al. (2009). Each time point was performed in triplicate. Using the GeneSpring GX 9.0 Analysis Platform (Agilent, Santa Clara, CA), microarray data was normalized and background corrected using robust multi-array average (RMA) analysis (Irizarry et al., 2003). Probe sets from the prion infection time course were filtered and statistically assessed as previously reported (Moody et al., 2009).

Quantitative RT-PCR

Total RNA was purified from brain tissue (4 mice/time point/infected state) using the Qiagen RNeasy Mini Kit protocol (Qiagen, Valencia, CA) and further treated with Turbo DNase I (Ambion, Houston, TX) to eliminate DNA contamination. cDNA from individual animals was synthesized in duplicate using the ImProm-II reverse transcription kit (Promega, Madison, WI) and an anchored T7-oligo(dT)V primer. Transcripts used for reference were initially identified from normalized microarray data as mRNAs that did not vary in expression in uninfected or infected animals. To assess the stability of reference transcripts, 2 independent quantitative real-time polymerase chain reaction (qRT-PCR) reactions were performed on each cDNA sample using 100 ng of total RNA, SYBRGreen reaction mix (BioRad, Hercules, CA), and appropriate reference primer sets (final concentration, 200 nM). Stability of reference transcripts, β -actin (*Actb*), and γ -synergisin (*Synrg*) was validated using the BestKeeper program (Pfaffl et al., 2004).

For target gene expression analysis, cDNA from two independent ImProm-II (Promega, Madison, WI) reactions were analyzed, giving a total of four reactions for each RNA sample. Reactions were performed using the iCycler real-time PCR detection system (BioRad, Hercules, CA) using the same cycling conditions for all reactions: 95°C for 3 min

followed by 50 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s. Amplification was immediately followed by a melt curve protocol, consisting of 30 s of 60°C, followed by 80 cycles that were 30 s long each with a 0.5°C increase in temperature at each step. Threshold values for threshold cycle (C_t) determination were generated by iCycler software.

To determine relative expression ratios for each sample, target RNA was first normalized to the reference RNA using the formula: $2^{(C_t \text{ internal standard} - C_t \text{ target})}$. Relative expression ratio (RER) values of the target genes were determined as previously described (Rotenberg et al., 2006). Statistical analysis of RER values was performed for uninfected against infected samples at 158 and 198 dpi timepoints with GraphPad Prism software using the unpaired two-tailed *t*-test function (GraphPad Software, La Jolla, CA).

Gene-specific primers used were: *ligp1* F 5'-CAGGACATCCGCCTTAAGTGT-3', *ligp1* R 5'-AGGAAGTAAGTACCCATTAGCCA-3'; *Irgm1* F 5'-TTTCATCAATGCACTTCGAGTCA-3', *Irgm1* R 5'-AATCCAGGTAAGTCCCACAGC-3'; *Gbp2* F 5'-GCAGCACCTTCATCTACAACAG-3', *Gbp2* R 5'-CACAAAGTTAGCGGAATCGTCTACC-3'; *Ifit3* F 5'-GACAGCAGTGAGAGAA GACAGAGG-3', *Ifit3* R 5'-TCTCCTTACTGATG ACCATCTGATAGC-3'; *Actb* F 5'-TATGGAATC CTGTGGCAT-3', *Actb* R 5'-TGTTGGCATAGA GGTCTT-3'; *Synrg* F 5'-TTGGTTCCAGATGCC TATAAGAA-3', *Synrg* R 5'-ACATCAGAATGGG ATAAGTTTAGC-3';

RESULTS

Interferon-Gamma-Induced Transcripts are Upregulated During Preclinical and Clinical RML Infection

Moody et al. (2009) previously described the identification of 164 transcripts differentially regulated during RML infection. Upon critical analysis, eight transcripts (*Ccl12*, *Cxcl10*, *ligp1*, *Ifi35*, *Irgm1*, *Gbp2*, *Gbp3*, *Gbp6*) were identified whose expression was classified as dependent on the cytokine IFN- γ . The expression of three additional transcripts (*Ifit3*, *Ifitm1*, *Rsad2*) was reliant on interferon-alpha/interferon-beta (IFN- α/β) and to a lesser extent IFN- γ (Table 1).

Based on the microarray data, each of the IFN- γ -induced transcripts were upregulated at both preclinical (158 dpi) and clinical (198 dpi) time points, with the exception of one transcript (*Ifitm1*) whose expression peaked prior to onset of clinical disease. Six of 11 IFN- γ -induced transcripts were more abundant at 158 dpi than at clinical disease, and 3 other IFN- γ -induced transcripts were similarly abundant at both preclinical (158 dpi) and clinical time points. The differential expression of five of the IFN- γ -induced transcripts in response to prion infection was observed by other groups, in addition to our own microarray analysis (Riemer et al., 2000, 2004; Baker & Manuelidis, 2003; Baker et al., 2004; Booth et al., 2004b; Xiang et al., 2004; Moody et al., 2009).

Validation of Microarray Data

The upregulation of 4 IFN- γ -induced transcripts, *Irgm1*, *ligp1*, *Gbp2*, and *Ifit3*, was confirmed with qRT-PCR. Relative expression ratios of *Irgm1*, *ligp1*, *Gbp2*, and *Ifit3* were

measured from both infected and control brain tissue at two time points during prion disease, as none of the transcripts tested showed upregulation in infected tissue at 108 dpi using microarray analysis. For all four transcripts, relative expression ratios with qRT-PCR showed statistically significant upregulation in infected tissue as compared to uninfected tissue (Figure 1). The results validate both the abundance values obtained from our microarray analysis (Table 1) and the upregulation of IFN- γ -induced transcripts during preclinical and clinical RML infection.

DISCUSSION

Several transcripts thought to be induced by IFN- γ were upregulated in response to RML infection in preclinical and clinically affected brain tissue. Many of these gene expression changes were noted elsewhere (Riemer et al., 2000, 2004; Baker & Manuelidis, 2003; Baker et al., 2004; Booth et al., 2004b; Xiang et al., 2004; Moody et al., 2009); however, emphasis has not been placed on their common means of induction. This study focused on and validated the upregulation of these transcripts during mouse-adapted prion disease and established a basis for future research into the role of IFN- γ in prion infection.

IFN- γ (also known as type II interferon) is an immunomodulatory cytokine that is a central mediator in host response to a variety of infectious agents. IFN- γ induces the expression of a specific set of genes through a distinct Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway (Schroder et al., 2004). Of the transcripts highlighted here, *Iigp1*, *Irgm1*, *Gbp2*, *Cxcl10*, and *Ifit3* were induced after IFN- γ stimulation in murine astrocytes (Halonen et al., 2006) and, in rat microglia, *Cxcl10* and *Gbp2* (Moran et al., 2004). IFN- γ is produced exclusively by activated T cells and natural killer cells. These cells infiltrate the brain only at the onset of clinical disease (185 dpi) during an intraperitoneally (ip)-administered RML infection (Lewicki et al., 2003), due to the maintained integrity of the blood–brain barrier during preclinical stages of prion disease. Early experiments failed to detect IFN- γ mRNA upregulation (Baker & Manuelidis, 2003; Campbell et al., 1994) or IFN- γ protein in the brains of prion-infected animals (Katz & Koprowski, 1968; Gresser et al., 1983). However, recent studies using highly sensitive immunobead detection systems demonstrated a significant increase of IFN- γ protein in the brains of prion-infected mice at clinical stage (Tribouillard-Tanvier et al., 2009).

Three major families of GTPases are regulated by interferons: the Mx proteins that are induced by IFN- α/β (type I interferons) (Staeheli & Haller, 1987), and the 65-kD guanylate-binding proteins (GBPs) and the p47 GTPases, both of which are induced by IFN- γ (Boehm et al., 1998). GTPases bind and hydrolyze GTP, enabling them to regulate many cellular functions. The immune function of GBP is unknown, but evidence suggests a role in resistance to viral infection (Anderson et al., 1999). Many studies described p47 GTPase expression in response to infection with protozoa, bacteria, and viruses for maintenance of both acute and chronic resistance to disease (Carlow et al., 1998; Collazo et al., 2002; Zerrahn et al., 2002). In this study, five IFN- γ -induced GTPases that are upregulated in response to RML infection were identified; three GBP (*Gbp2*, *Gbp3*, *Gbp6*) and two p47 GTPases (*Iigp1*, *Irgm1*) (Table 1).

Three additional transcripts known to be induced by IFN- γ were also identified : *Ccl12*, *Cxcl10*, and *Ifi35*. *Ccl12* encodes a cytokine that is known to elicit chemotactic movement from eosinophils, monocytes, and lymphocytes (Jia et al., 1996). The protein product of *Cxcl10* was shown to possess chemoattraction for macrophages, T cells, and natural killer cells, suggesting a role for immune cell recruitment to areas of inflammation (Dufour et al., 2002). While cellular actions of IFI35 are not fully understood, there is evidence toward an anti-apoptotic role (Zhang et al., 2007). Collectively, these transcripts encode for cytokines that attract immune mediators to sites of distress and possibly inhibit apoptosis.

Three transcripts whose expression can be induced by either type I or type II interferons were also identified in our analysis: *Ifit3*, *Ifitm1*, and *Rsad2*. While these transcripts are more reliant on type I interferons for induction, it is possible that their expression is linked to the signaling cascade responsible for the other IFN- γ -stimulated transcripts described here. *Ifit3* encodes for a GTPase with uncharacterized antiviral properties (Saha & Rangarajan, 2003). The protein product of *Ifitm1* plays a role in the antiproliferative activity of interferons (Deblandre et al., 1995). *Rsad2* (a.k.a. viperin) encodes for a product which possesses antiviral properties against several viruses (Helbig et al., 2005; Riviuccio et al., 2006; Jiang et al., 2008).

The molecular events that occur in the brain during prion infection are not well understood. The elucidation of these events, with a focus on how the body responds to infection, enables investigators to both better understand the pathology of prion disease and provide targets for future diagnostics. Evidence indicates that at the transcriptional level there may be a role for IFN- γ -inducible gene products in prion disease. As IFN- γ was recently detected in the brains of prion-infected mice, these “IFN- γ -inducible” transcripts provide targets for future IFN- γ -related studies. Alternatively, the regulation of these “IFN- γ -inducible” transcripts may be regulated via an IFN- γ -independent mechanism. Currently there are ongoing experiments analyzing IFN- γ -stimulated protein expression in preclinical and clinically infected brain tissue, as well as directly testing the hypothesis that IFN- γ is necessary for the progression and presentation of prion infection.

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FIGURE 1.

Confirmation of microarray data from four IFN- γ induced transcripts with qRT-PCR. RML-infected brain homogenate obtained at one preclinical time point (158 dpi) and at clinical infection (198 dpi). cDNA was produced from total RNA from four infected or four control mice and subjected to qRT-PCR to assess the abundance of *Irgm1*, *ligp1*, *Gbp2*, or *Ifit3*. Relative expression ratios were generated using the Pfaffl method after qRT-PCR. *Actb* and *Synrg* were used as endogenous controls for normalization. At both time points and for all transcripts, transcripts were statistically upregulated in RML-infected brain tissue over uninfected controls. Significant differences indicated by * $p < .05$, ** $p < .01$.

TABLE 1

Transcripts Induced by IFN- γ in Brain Tissue During RML Infection

Probe set ID	Gene symbol	Gene title	108 dpi	158 dpi	198 dpi	Interferon dependence	Publications
<i>65-kDa guanylate-binding proteins (GBP_s)</i>							
1418240_at	Gbp2	guanylate nucleotide binding protein 2	1.3	3.0	2.2	IFN- γ	6,7
1418392_a_at	Gbp3	guanylate nucleotide binding protein 3	1.2	1.8	1.7	IFN- γ	7
1425156_at	Gbp6	guanylate binding protein 6	1.1	1.7	1.5	IFN- γ	7
<i>p47 GTPases</i>							
1419043_a_at	Iigp1	interferon inducible GTPase 1	1.2	2.7	2.4	IFN- γ	1,7
1418825_at	Irgm1	immunity-related GTPase family, M	1.2	2.0	2.3	IFN- γ	7
<i>Non-GTPases</i>							
1419282_at	Ccl12	chemokine (C-C motif) ligand 12	1.1	2.6	2.7	IFN- γ	6,7
1418930_at	Cxcl10	chemokine (C-X-C motif) ligand 10	1.2	4.4	2.7	IFN- γ	1,2,3,5,6,7
1424617_at	Ifi35	interferon-induced protein 35	1.1	1.6	1.6	IFN- γ	7
1449025_at	Ifit3	interferon-induced protein with tetratricopeptide repeats 3	1.2	2.8	2.8	IFN- α/β > IFN- γ	4,5,7
1421009_at	Rsad2	radical S-adenosyl methionine domain containing 2	1.3	2.1	2.2	IFN- α/β > IFN- γ	7
1424254_at	Ifitm1	interferon induced transmembrane protein 1	-1.1	1.7	1.1	IFN- α/β > IFN- γ	7

Note. Fold change versus control values is shown for three time points during disease. Transcripts previously described as upregulated during prior infection are indicated.

Sources:

- ¹ Riemer et al. (2000)
- ² Baker and Manuelidis (2003)
- ³ Baker et al. (2004)
- ⁴ Booth et al. (2004b)
- ⁵ Riemer et al. (2004)
- ⁶ Xiang et al. (2004)
- ⁷ Moody et al. (2009).