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14. ABSTRACT

Thegoal of this project is to identify pathways and networks of genes and proteins perturbed by prion replication.The unusualnature of prion disease prompted a systems approach to identify networks specifically perturbed by prion infections and to determine which perturbations are essential for various aspects of the disease. We previously tracked changes in gene expression ninbrain and spleen for two different prion strains and five different lines of mice over their entire incubation periods. We have successfully infected CNS stem cell containing neurosphere cultures with the Rocky Mountain Laboratory (RML) prion strain. Neurosphere lines have been produced from the same mouse strains used for our invivo studies. Differential gene and protein expression in these cells will aid in identifying genes directly involved in prion replication and lead to the identification of markers for prion infected individuals. We also are developing neurosphere cultures as a sensitive, rapidbioassay for mouse,bovine,and human prions.

15. SUBJECT TERMS
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Early Host Responses to Prion Infection: Development of In Vivo and In Vitro Assays

George A. Carlson, PhD, Principal Investigator
Leroy E. Hood, MD, PhD, Co-Investigator

Annual report: September 1, 2005 to August 31, 2006

INTRODUCTION

A misfolded form of prion protein (PrP^{Sc}) is the functional component of infectious prions and is derived by posttranslational templating of the normal PrP^{C} isoform (Prusiner, 1998). A hallmark of prion disease is its long incubation period, which is the interval between inoculation and onset of clinical signs of neurological dysfunction. In rodents, the earliest detectable indicator of prion infection is a rise in infectivity. The ability to infect experimental animals is a more sensitive assay for prions than commonly used methods to detect PrP^{Sc} by immunoblotting, which generally requires between 10^5 and 10^7 infectious units (IU) per ml. New technologies, such as the conformation dependent immunoassay (Safar et al., 2000; Safar et al., 2005), are providing dramatic increases in sensitivity and further improvements in detection of PrP^{Sc} are likely. However, specific changes in host biology that accompany, or even precede, conversion of PrP^{C} to PrP^{Sc} may provide more sensitive indicators of prion exposure.

Based on the hypothesis that a variety of intracellular and intercellular systems are perturbed at both the protein and gene transcription levels by prion replication, we have applied a discovery-based approach to identify changes induced by prions that can be used as a signature for prion-infected individuals. To identify a gene expression signature specific to prion-infected individuals, we used Affymetrix chips to search for changes in mRNA expression that showed overlap in different mouse strain-prion strain combinations. This work using mice is nearing completion. One impediment to application of arrays to prion disease and to mechanistic analysis of genotype-agent strain interactions has been the lack of a versatile tissue culture assay for prion infection. The ability to infect cells derived from mice of any genotype or from humans would provide a new tool for dissecting prion disease and for validating in vitro assays for infectivity. With support from the Department of Defense National Prion Research Program, we have successfully infected central nervous system (CNS) stem cell (SC) lines from a variety of normal and transgenic mice with mouse scrapie prions. One goal of this project is to develop CNS-SC lines from transgenic mice over-expressing human or bovine PrP that can be used as in vitro bioassays for human and bovine prions. Current bioassays for human or bovine prions require inoculation of transgenic mice and waiting for disease to develop. Although requiring up to two years for definitive results, bioassays in mice are more sensitive than current screening tests based on detection of misfolded, proteinase-resistant PrP. CNS-SC lines also will facilitate our global microarray and proteomics approaches to identify gene expression signatures specific to prion-infected individuals before they are clinically ill. The ultimate goal is to develop sensitive blood tests for humans and animals incubating infectious prions.

BODY

Progress towards completing each specific task in the Statement of Work are indicated in **bold type** below:

Task 1. Determine whether there are specific changes in mRNA and protein expression profiles following prion infection in neurosphere (CNS-stem cell) cultures. (Months 1-12) **In progress. Results for two independent neurosphere cultures from Tg(MoPrP-A)4053 infected with**

the RML strain of prions have been obtained. A subset of the genes differentially expressed in prion infected mice are found in infected neurospheres compared with those exposed to normal brain homogenate.

Task 2. Compare results from neurosphere cultures to results obtained using prion infected mice from peripheral blood cells, spleens, and brains using the same mouse strain-prion strain combinations. Gene expression signatures potentially specific to prion-infected individuals will be identified. (Months 3-13) **Unexpectedly, infection of neurospheres with prion strains other than RML has proven to be problematic. We are modifying culture conditions to allow infection with the 301V prion isolate; this strain was used in our in vivo studies.**

Task 3. Validate putative prion-specific gene expression signatures as a blood test to identify prion-infected mice before clinical signs of disease appear. (Months 13-18)
Work using brain-specific differentially expressed genes (DEGs) identified in our in vivo studies is proceeding. DEGs predicted by massively parallel signature sequencing to encode proteins solely expressed in the CNS and predicted to be secreted into blood are under analysis. Results from our CNS stem cell cultures will be incorporated into these studies in the coming months. A procedure for intracellular proteome analysis also was developed.

Task 4. Establish neurosphere cultures from transgenic mice over-expressing human PrP (three lines) or bovine PrP (one line). (Months 1-4) **Completed. The cell lines are now in the BSL-3 laboratory of our collaborator, Dr. Stanley B. Prusiner, Director of the Institute for Neurodegenerative Diseases (IND), University of California, San Francisco (UCSF).**

Task 5. Determine whether neurosphere lines from transgenic mice over-expressing human or bovine PrP can be infected with human and bovine prions. (Months 4-12) **Attempts to infect neurosphere lines with human or bovine prions is in progress. As we found for mouse prion strains other than RML, this is proving difficult.**

Task 6. Establish methods for sensitive bioassay of prions in tissue culture using neurospheres from transgenic mice overexpressing mouse PrP. (Months 1-18) **A sensitive assay that recapitulates genetic susceptibility in vivo has been developed for RML prions. We are working to develop methods to allow detection of other mouse prion strains or prions from other species.**

Task 1. While CNS stem cell lines were being established from several different mouse strains in Dr. Carlson's laboratory at McLaughlin Research Institute (MRI), Dr. Inyoul Lee at the Institute for Systems Biology (ISB) performed a pilot study on two of our mouse CNS stem cells derived from two individual FVB Tg(MoPrP-A)B4053 mice (designated Tg1 and Tg2). These were inoculated with brain homogenates obtained from both normal and RML prion-infected mice, and then cultured up to five passages. Thirty two total RNA samples from the normal and prion-infected CNS stem cells were prepared at 2, 6, 12, 21, and 36 days post inoculation during the first two passages, and then at 65, 82, and 91 days at the third, fourth, and fifth passages post inoculation, respectively. Transcript abundances in these samples were then quantified using Affymetrix GeneChip Mouse Genome 430 2.0 arrays. We are currently analyzing these expression data to assess their quality and reproducibility and to build networks based on DEGs found in the CNS stem cells for comparison with those from mice.

Task 2. CNS neurosphere lines have been produced from FVB/NCr, C57BL/6J, B6.I, FVB.129-*Prnp*^{tm1Zrch} (PrP-null) mice and those expressing PrP have been infected with the Rocky Mountain Lab (RML) prion strain to compare DEGs in neurospheres, which don't show obvious cytopathic effects prior to differentiation, and DEGs in the mice shown in Table 1, for which we have completed analysis of dynamic changes in DEGs over the course of disease. Only PrP 0/+ neurospheres remain to be made for this study.

Table 1. Host-prion combinations for global analysis of networks perturbed by prion replication

Mouse Strain	*Prnp* Genotype	Prion Strain	Incubation Time (d)
C57BL/6J	*a/a*	RML	~150
C57BL/6J	*a/a*	301V	~260
B6.I-1	*b/b*	RML	~350
B6.I-1	*b/b*	301V	~120
FVB. *Prnp*^{tm1}	*0/0*	RML	No illness
(FVB x FVB. *Prnp*^{tm1})F1	*a/0*	RML	~400
FVB/NCr	*a/a*	RML	~150
FVB.Tg(MoPrP)B4053	>30 *a*	RML	~50

To complete our comparison, we need to be able to infect neurospheres with the mouse adapted BSE strain 301V (and other strains) as well as with RML. This has proven difficult. Using the same conditions of that allowed infection with RML prions, we tested the ability of ME7, 301V, 22A, and 87V prion strains to infect neurospheres from *Prnp*^{a} and *Prnp*^{b} transgenic and non-transgenic mice and were unsuccessful. It is clear that detection of infection with these strains will be less straightforward than for RML. We are now evaluating three approaches to increase the numbers of prion strains that can be infected with prions: modifications to culture conditions, to the prion isolates, and to the neurospheres themselves.

Task 3. A data integration scheme has been developed in order to identify potential diagnostic markers using various sources of data. Our preliminary results were obtained from our mouse transcriptome data by applying the four steps shown in Figure 1. This data integration scheme will be applied to data collected from the CNS stem cells. As the first step, we identified 1127 DEGs showing shared dynamic patterns of differential expression in all the prion-infected brain samples of the five mouse strain/prion strain combinations. For the second step, we found a subset of those 1127 DEGs that were mainly expressed several brain subregions by integrating transcriptome libraries of Massively Parallel Signature Sequencing (MPSS) from 40 mouse tissues (http://www.ncbi.nlm.nih.gov/projects/geo/info/mouse-trans.html). From this analysis, 100 transcripts (out of 1127) were predicted to be nervous system-specific transcripts.

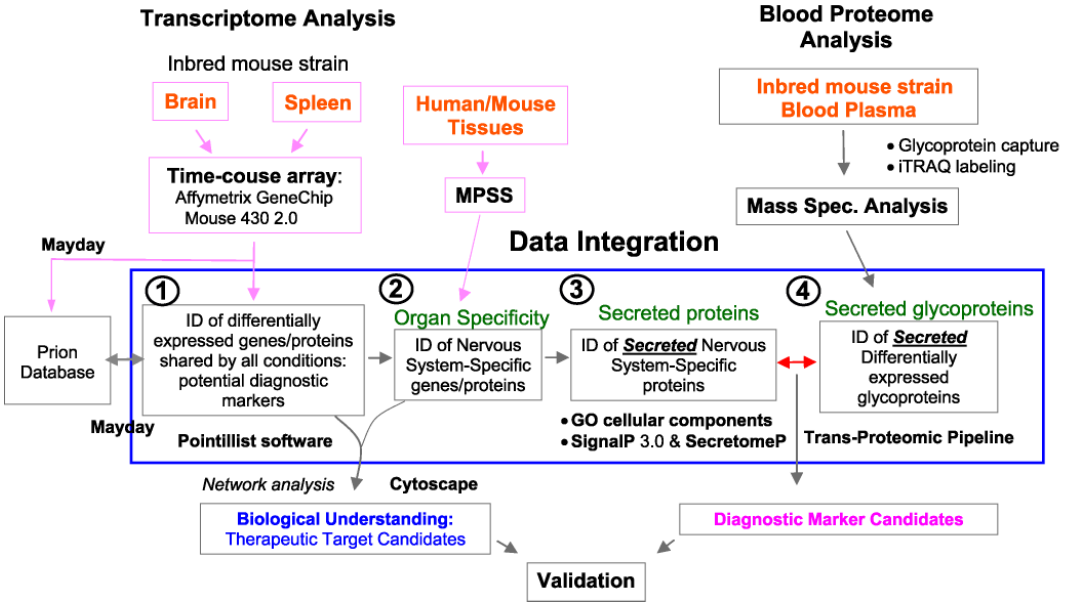

Figure 1. Data Integration Strategy.

The third step, further focused on nervous system-specific and secreted diagnostic markers. We selected 49 transcripts of 100 nervous system-specific DEGs by integrating secretion information from GO cellular components and predictions of potential secretion into the blood by the programs SignalP (http://www.cbs.dtu.dk/services/SignalP/) and SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP-1.0/). (see Figure 1).
For the fourth step, we carried out serum proteomic analysis. As a result, we found 26 proteins differentially expressed between the serum samples prepared from normal and RML-infected C57BL/6J mice. Figure 2 shows expression profiles of the 11 out of 26 differentially expressed proteins from RML-infected C57BL/6J mice. Tissue expression patterns of 13 of these 26 proteins were found to be restricted to nervous system through literature search or comparison with mouse MPSS data from 40 tissues.
In parallel, we are evaluating the potential of these candidates for diagnostic blood markers by testing whether abundance of some of these candidate proteins can be distinctly monitored through Western Blot analysis or electrochemiluminescence-based immunoassay on serum samples from normal and prion-infected mice.

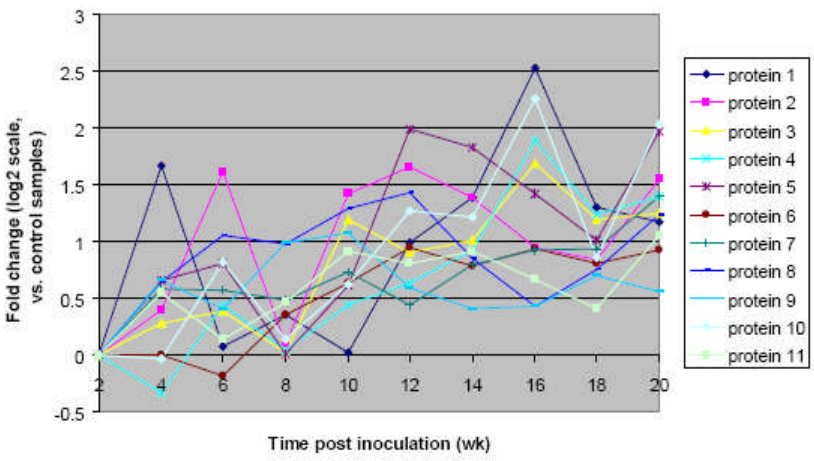

Figure 2. The subtractive expression profile of eleven serum proteins upon prion infection. The abundance change (fold change in log2 scale) of 11 serum proteins between normal and RML-infected C57BL/6J mouse serum samples are shown across the whole time course of incubation time.

Drs. Lee and Hyuntae Yoo at ISB also have developed a streamlined procedure for intracellular proteome analysis of CNS neurosphere cells. They used brain homogenate samples from normal C57BL/6J mice as an equivalent system for establishing the proteomic analysis tools. The focus was on optimizing the following experimental techniques as important steps in analyzing the intracellular proteome: a) protein extraction from the tissue samples; b) tryptic digestion of the proteins in preparation for shotgun proteomic analysis; c) stable-isotope labeling of the tryptic peptides for quantification of proteins from multiple samples; d) LC/MS/MS analysis of the isotope-labeled peptide mixtures for identification and quantification of the intracellular proteins; and e) computational analysis on the proteomics datasets with the various computational tools developed at ISB. These software tools consist of programs for the entire proteomic production pipeline and for evaluating the data quality (http://tools.proteomecenter.org/software.php).

Task 4. We have isolated neurosphere lines from transgeneic mice expressing either the 129M or 129V allotypes of human PrP-- Tg(HuPrP-129M)B440 and Tg(HuPrP-129V)152. These alleles determine susceptibility to sporadic and iatrogenic CJD, as well as to variant CJD caused by BSE prions from cattle; all cases of vCJD to date have been found in 129M homozygous individuals. We have also produced a line from Tg(MHu2M-165V,167Q)22372 mice that are highly susceptible to infection with human prions. Finally, we have isolated a line from Tg(BoPrP)E4092 mice that overexpress bovine PrP and that can be infected with BSE prions.

Task 5. Work is underway in the laboratory of our collaborator, Dr. Stanley B. Prusiner, to infect the above lines (Task 4) with human and bovine prions. A BSL-3 facility is required for this work and is available in Dr. Prusiner's lab at UCSF's IND. So far, we have been unsuccessful.

Task 6. As described in our May 2006 annual report for National Prion Research Program Grant DAMD17-03-1-0321, we have been able to detect RML prions diluted 10^{-8}. At passage 1, only rare PK-resistant PrP^{Sc}-positive colonies were detected in cell lines infected with high dilutions of RML prions. On subsequent passages, the level of PK-resistant PrP^{Sc} produced by cultures exposed to high dilutions of prions increased. Importantly, cultures exposed to RML diluted 10^{-8}, which showed little PrP^{Sc} at passages 1 and 2, showed many positive colonies by passage 3. These findings demonstrate that prions in Tg4053 neurosphere cultures replicate and spread from cell to cell. As noted above, infecting with prion strains other than RML has proven to be problematic. An alternative approach for detection of exposure to prions has been suggested by our studies comparing differentiation of stem cells from prion-infected and noninfected neurosphere cultures.

Removal of growth factors and addition of retinoic acid and forskolin induced loss of the stem/progenitor cell marker nestin that was comparable in infected and non-infected cultures. In contrast, the cellular composition following differentiation differred dramatically between infected and non-infected cultures. For example, far fewer GABA-staining neurons (GABA and neurofilament H-positive) were produced from prion infected cultures than from non-infected neurospheres. Total length and number of cellular processes were guantified using MetaMorph image analysis software as was the level of fluorescent staining for GABA. The results are shown in Figure 3. The lesser numbers and shorter cellular processes suggest that differentiation reveals pathogenic effects of prion replication not readily observable in neurosphere cultures; this augurs well for development of in vitro models to study prion pathogenesis in cell culture. In contrast to our results for GABAergic neurons, more astrocytes are produced from infected cultures than non-infected ones. The rapid disappearance of nestin and appearance of markers of differentiated cells also may indicate a difference in the cellular

composition, perhaps in lineage-committed progenitor cells, between infected and non-infected neurospheres that could be used as a marker for infection.

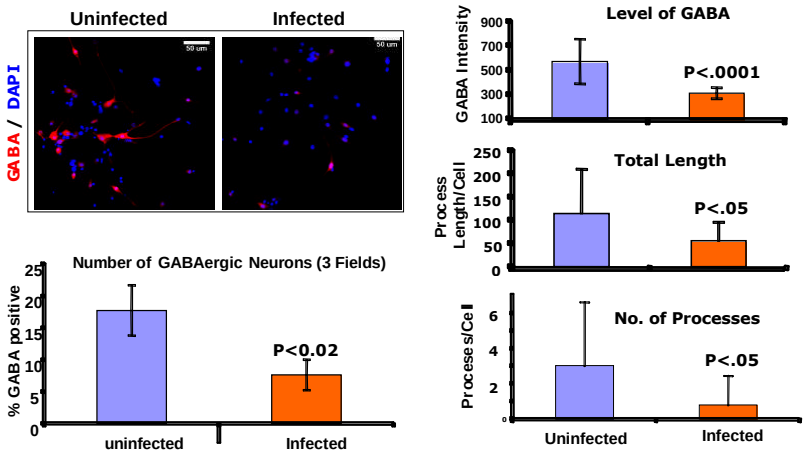

Figure 3. RML-prion infected neurosphere cultures produce fewer GABA-positive cells containing less GABA and having shorter and fewer cellular processes than uninfected cultures. RML-infected (passage 10 post-infection) and control Tg4053 neurospheres were dissociated and cultured for 24 hr on poly-D-lysine and lamin coated plates in the presence of retinoic acid and forskolin. GABA staining is in red with nuclei marked by DAPI. Percentage (± SD) of positive cells in three fields were counted. Intensity of GABA staining, number of processes per cell and average total length of the processes per cell were determined by counting 80-100 individual cells.

KEY RESEARCH ACCOMPLISHMENTS

Produced stem cell neurosphere lines from the mouse strains used for dynamic analysis of gene expression following prion infection in vivo.

Identified DEGs specific to RML prion infection of Tg(MoPrP-A)4053 neurospheres.

Developed a data integration strategy for identification and validation of prion disease-specific biomarkers.

Developed a streamlined procedure for intracellular proteome analysis of cells from CNS stem cell containing neurosphere cultures.

Isolated neurosphere lines from transgenic mice expressing human or bovine PrP.

REPORTABLE OUTCOMES, YEAR 1.

Astracts for our presentations at the Prion 2006 International Congress (Turin) and at the Fourth International Conference on Pathways, Networks, and Systems (Mykonos) are appended.

CONCLUSIONS

We have infected CNS stem cell containing mouse neurosphere cultures with prions. The ability to readily produce prion-infectable cell lines from any strain or transgenic line of mice offers the unprecedented opportunity to explore the genetics of disease susceptibility in culture. Work to extend these studies to include prion strains in addition to RML and to develop sensitive bioassays for prions from other species is in progress. If successful in establishing infected lines with various prion strains, these could serve a reference cultures that could be shared among laboratories. Application of systems biology to these cultures provides a powerful tool to identify differentially expressed genes whose encoded proteins may be necessary for prion replication. A subset of these disease-specific proteins in blood may identify prion-infected individuals.

SYSTEMS APPROACH TO PRION PATHOGENESIS

I. Lee[1], D. Hwang[1], H. Yoo[1], D. Baxter[1], N. Gehlenborg[1], R.K. Giri[2], R. Kumar[2], B. Ogata[1], M. Orr[2], R. Pitstick[2], D. Spicer[2], R. Young[2], S.J. DeArmond[3], L.E. Hood[1], G.A. Carlson[2]

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A systems view of disease attempts to understand disease initiation and progression in terms of specific-perturbations and their dynamic transitions. The unusual nature of prion disease and the variety of strains and pathologies prompted exploration of a systems approach to identify networks perturbed by infection and to determine which perturbations are essential for various aspects of pathogenesis. Using the Affymetrix GeneChip mouse array 430 2.0, we tracked changes in gene expression for two prion strains (RML and 301V) and five lines of mice over their entire incubation periods; PrP null mice also were inoculated. Differentially expressed genes (DEGs) with consistent temporal patterns across multiple mouse-prion groups were considered likely to be associated with fundamental prion disease processes. However, the goal of this study was not to compile a list of DEGs, but rather to integrate multiple types of data to provide a new perspective on disease for hypothesis building and testing. Array data were used in conjunction with gene ontology, protein interaction and gene regulatory databases to construct hypothetical pathways and gene regulatory networks associated with major pathological events, including glial activation, synaptic degeneration, cell death, and protein degradation. The mismatch between rate of PrP^{Sc} accumulation and disease onset and differences in DEGs in specific agent-host combinations were used to formulate and test hypotheses on the involvement of specific pathways in disease. For example, lack of changes in a pathway involved in generation of reactive oxygen species (ROS) in transgenic mice with short incubation times suggested that ROS might not be an essential component of neurological dysfunction. In accord with this prediction, overexpression of SOD1, shown to be effective in our Alzheimer's disease models, had no effect on prion incubation time. Data and analysis tools are available on the internet in our searchable Prion Disease Database.

4th International Conference on Pathways, Networks, and Systems, October 2006, Mykonos

GENETIC DIFFERENCES IN SUSCEPTIBILITY OF NEUROSPHERE CULTURES TO PRION INFECTION

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Prion protein genotype (*Prnp* in mice) is the most important determinant of susceptibility and disease phenotype in every known prion disorder. Background genes other than *Prnp* also significantly influence incubation time in mice and chromosomal locations have been determined for several of these modifier loci. Due to the limited genetic diversity and aneuploidy of prion infectible cell lines and the consequent reliance on expensive and time-consuming bioassays in mice, there is only limited understanding of the cellular mechanisms underlying genetic differences in susceptibility. CNS-stem cell-containing neurosphere cultures were produced to test their suitability for studying the genetics of susceptibility to prions. Efficiency of infection, spread from cell to cell, and rate of prion replication can be discriminated in neurosphere cultures using filter-based immmunostaining for PK-resistant PrP^{Sc} or conformation-dependent immunocytochemistry (Giri et al., Proc Natl Acad Sci USA 2006,103:3875). The RML strain of prions produces short incubation times in mice expressing the *a* allele of *Prnp* and long incubation times in $Prnp^{b}$ mice. Neurosphere lines were isolated from C57Bl/6J (B6), B6.I-$Prnp^{b}$, Tg(MoPrP-A)B4053, and Tg(MoPrP-B)C2091 mice; the transgenic lines express comparable levels of the alternative PrP allotypes. Infection could be established at higher dilutions of RML prions and spread of infection through the cultures was more rapid in neurosphere lines expressing PrP-A than those expressing PrP-B, reflecting the short and long incubation times of the mice from which they were derived. For example, by passage 3, most neurosphere colonies were PrP^{Sc}-positive in TgB4053 cells incubated with isolate diluted 10^{-8}. The PrP-B producing neurospheres were far less sensitive to infection, with de novo PrP^{Sc} production achieved only by dilutions of 10^{-4} to 10^{-5}. To our knowledge, this is the first demonstration of allelic differences in prion susceptibility in mice modeled in tissue culture.

Prion 2006, October 2006, Turin

SYSTEMS BIOLOGY APPROACH TO PRION DISEASE PATHOGENESIS

I. Lee[1], D. Hwang[1], H. Yoo[1], D. Baxter[1], N. Gehlenborg[1], R.K. Giri[2], R. Kumar[2], B. Ogata[1], M. Orr[2], R. Pitstick[2], D. Spicer[2], R. Young[2], S.J. DeArmond[3], L.E. Hood[1], G.A. Carlson[2].

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A systems view of disease attempts to understand disease initiation and progression in terms of specific-perturbations and their dynamic transitions. The unusual nature of prion disease and the variety of strains and pathologies prompted exploration of a systems approach to identify networks perturbed by infection and to determine which perturbations are essential for various aspects of pathogenesis. Using the Affymetrix GeneChip mouse array 430 2.0, we tracked changes in gene expression for two prion strains (RML and 301V) and five lines of mice over their entire incubation periods; PrP null mice also were inoculated. Differentially expressed genes (DEGs) with consistent temporal patterns across multiple mouse-prion groups were considered likely to be associated with fundamental prion disease processes. However, the goal of this study was not to compile a list of DEGs, but rather to integrate multiple types of data to provide a new perspective on disease for hypothesis building and testing. Array data were used in conjunction with gene ontology, protein interaction and gene regulatory databases to construct hypothetical pathways and gene regulatory networks associated with major pathological events, including glial activation, synaptic degeneration, cell death, and protein degradation. These pathways were integrated with temporal changes in regional PrP^{Sc} distribution, pathology, and regional gene expression among the different host-agent combinations. The mismatch between rate of PrP^{Sc} accumulation and disease onset and differences in DEGs in specific combinations were used to formulate and test hypotheses on the involvement of specific pathways in disease. For example, lack of changes in a pathway involved in generation of reactive oxygen species (ROS) in transgenic mice with short incubation times suggested that ROS might not be an essential component of neurological dysfunction. In accord with this prediction, overexpression of SOD1, shown to be effective in our Alzheimer's disease models, had no effect on prion incubation time. Data and analysis tools are available on the internet in our searchable Prion Disease Database.

Prion 2006, October 2006, Turin