Pathogenic and nonpathogenic hantaviruses differentially regulate endothelial cell responses

Erika Geimonen*†‡, Sherry Neff*†‡, Tracy Raymond†, Salih S. Kocer§, Irina N. Gavrilovskaya*†, and Erich R. Mackow*†‡§¶

Departments of *Medicine and †Molecular Genetics and Microbiology, §Molecular Cell Biology Program, State University of New York, Stony Brook, NY 11794; and ‡Northport Veterans Administration Medical Center, Northport, NY 11768

Edited by Peter Palese, Mount Sinai School of Medicine, New York, NY, and approved July 15, 2002 (received for review May 17, 2002)

Hantaviruses cause two human diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Hantaviruses infect human endothelial cells but cause little or no damage to the infected endothelium. We analyzed with Affymetrix DNA Arrays (Santa Clara, CA) the endothelial cell transcriptional responses directed by hantaviruses associated with HPS [New York-1 virus (NY-1V)], HFRS [Hantaan virus (HTNV)], or by a hantavirus not associated with human disease [Prospect Hill virus (PHV)]. Hantavirus infections induced 117 cellular genes and repressed 25 genes by >3-fold, 4 days postinfection (p.i.). Although >80% of cells were infected by each virus 1 day p.i., PHV induced or repressed 67 genes at this early time compared with three genes altered by HTNV or NY-1V. The early high-level induction of 24 IFN-stimulated genes by PHV (4- to 229-fold) represents a fundamental difference in the temporal regulation of cellular responses by pathogenic and nonpathogenic hantaviruses. Because all hantaviruses induced >23 IFN-stimulated genes at late times p.i., pathogenic hantaviruses appear to suppress early cellular IFN responses that are activated by nonpathogenic hantaviruses. At late times p.i., 13 genes were commonly induced by HTNV and NY-1V that were not induced by PHV. In contrast to NY-1V, HTNV uniquely induced a variety of chemokines and cell adhesion molecules (i.e., IL-8, IL-6, GRO-β, ICAM), as well as two complement cascade-associated factors that may contribute to immune components of HFRS disease. NY-1V failed to induce most cellular chemokines directed by HTNV (3/14) or genes primarily activated by NF-κB. However, NY-1V uniquely induced β3 integrin-linked potassium channels, which could play a role in HPS-associated vascular permeability. These studies provide a basic understanding of hantavirus-directed cellular responses that are likely to differentiate pathogenic and nonpathogenic hantaviruses, contribute to HFRS and HPS pathogenesis, and provide insight into disease mechanisms and potential therapeutic interventions.

Hantaviruses are enveloped negative-stranded RNA viruses that are present worldwide. Each hantavirus persistently infects a primary small mammal host without apparent disease, and hantaviruses are spread to humans through the inhalation of aerosolized excreted virus (1). Hantaviruses predominantly infect endothelial cells lining the vasculature; however, there is no apparent damage to hantavirus-infected endothelial cells (2–5). In humans, hantaviruses cause two diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Both diseases are characterized by altered vascular permeability and acute thrombocytopenia and, in HPS cases, acute pulmonary edema is observed (4, 6–8). During HPS, there is little immune cell recruitment to the site of infection, whereas immune responses and immune complexes have been implicated in the HFRS disease process (4, 6–10). Currently there is no understanding of how hantaviruses cause HPS or HFRS diseases or why specific hantaviruses have primarily pulmonary or renal disease manifestations. However, hantavirus interactions with endothelial cells are likely to play a central role in viral pathogenesis.

β3 integrins confer cell susceptibility to HFRS- and HPS-causing hantaviruses but not to hantaviruses that are nonpathogenic (11–13). The use of β3 integrins by only pathogenic hantaviruses suggests one possible link between hantavirus endothelial cell interactions and disease (11, 14, 15). However, the fact that both pathogenic and nonpathogenic hantaviruses infect, but do not disrupt the vascular endothelium suggests that endothelial cell responses to specific hantaviruses are central to the development of HPS and HFRS diseases. The endothelium forms the primary barrier within the vasculature, and dysregulation of endothelial cell functions can cause a wide variety of vascular effects that lead to changes in vascular permeability or hemorrhage (14, 16–18). The vascular endothelium also directs immune responses through the induction of cytokines, chemokines, and cellular receptors that recruit or activate immune cells (19–22). As a result, hantavirus dysregulation of normal endothelial cell responses could broadly affect vascular permeability through both endothelial and immune cell responses, directing a multifactorial disease process.

The ability of pathogenic and nonpathogenic hantaviruses to alter endothelial cell responses is virtually unknown. In this study, we used Affymetrix DNA Array (Santa Clara, CA) analysis of cellular mRNA levels to investigate changes in endothelial cells induced by pathogenic hantaviruses and compared these responses with those induced by nonpathogenic hantaviruses. We observed >3-fold changes in the transcription of 142 endothelial cell genes present on a chip library of approximately 12,000 known human genes. Striking differences in the early induction of IFN-stimulated genes (ISGs) by pathogenic and nonpathogenic hantaviruses were observed and verified by quantitative real-time PCR. Pathogenic hantaviruses were also found to induce specific genes with potential roles in pathogenesis, and as a result these data provide an essential understanding of hantavirus–cell interactions that are likely to contribute to HFRS and HPS diseases.

MICROBIOLOGY

Materials and Methods

Cell and Virus. HPS-associated New York-1 virus (NY-1V), HFRS-associated Hantaan virus (HTNV), and Prospect Hill virus (PHV), which is not associated with human disease, were used in these studies. Biosafety level 3 facilities were used for NY-1V and HTNV cultivation. Human umbilical vein endothelial cells were purchased from Clonetics (San Diego) and grown in EBM-2 (Clonetics) supplemented with 0.1% endothelial cell growth factor. HTNV (76–118), NY-1V, and PHV were cultivated as previously described on VERO E6 cells, and viral titers used for these experiments were $\approx 5 \times 10^5$ (1). Endothelial cells (8×10^4) were infected in wells of a six-well plate at a multiplicity of infection (MOI) of 1 for DNA array and real-time PCR analysis. Cells were collected for RNA preparation 1 or 4 days postinfection (p.i.) with HTNV, NY-1V, or PHV or after mock infection of monolayers. Viral titers were determined by focus assay, and the number of infected cells 1 day

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: HFRS, hemorrhagic fever with renal syndrome; HPS, hantavirus pulmonary syndrome; ISGs, IFN-stimulated genes; NY-1V, New York-1 virus; HTNV, Hantaan virus; PHV, Prospect Hill virus; MOI, multiplicity of infection; p.i., postinfection; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; VEGF, vascular endothelial growth factor; TRAF, tumor necrosis factor receptor-associated factor.

¶To whom correspondence should be addressed. E-mail: EMackow@mail.som.sunysb.edu.

p.i. was quantitated by immunoperoxidase staining of the hantavirus nucleocapsid protein by using polyclonal rabbit antinucleocapsid sera (23). Monolayers that were 80–90% infected 1 day p.i. were used for DNA array analysis.

RNA Preparation and Microarray Analysis. Total RNA was extracted from cells by using the RNeasy kit (Qiagen, Chatsworth, CA), according to the manufacturer's protocol. Reverse transcription, second-strand synthesis, and probe generation were accomplished by standard Affymetrix protocols. The Human Genome HG-U95Av2 GeneChip (Affymetrix), containing ≈12,000 known genes, was hybridized, washed, and scanned according to Affymetrix protocols within the Stony Brook Affymetrix Core facility. Changes in cellular mRNA levels after hantavirus infection were compared with mRNA levels in mock-infected controls that were identically plated, treated, and incubated in the absence of virus. Affymetrix MICROARRAY ANALYSIS SUITE, Ver. 4.0.1, was used to normalize and scale results and compare viral responses to those of controls. The program clusters increases or decreases and hierarchically presents levels as the fold change relative to control.

RT–PCR and Quantitative Real-Time PCR Analysis. Selected genes identified by DNA array analysis were also analyzed by quantitative real-time PCR to verify transcriptional responses. Total RNA (1 μg) was reverse transcribed by using Oligo-p$(dT)_{15}$ primers and the 1st Strand cDNA Synthesis Kit (Roche). cDNA (2 μl of a 1:100 dilution) was subjected to real-time PCR on the LightCycler (Roche Diagnostics) by using the FastStart DNA Master Mix, SYBR Green I (Roche), containing 4 mM $MgCl_2$ and 0.5 μM each primer. Results from virus-infected samples were compared with mock-infected mRNA levels. LightCycler PCR conditions were 95°C for 5 min followed by 40 cycles of: 95°C for 15 sec, 55–62°C for 5 sec, and 72°C for 5 sec. Fluorescence changes were monitored after every cycle, and melting curve analysis was performed at the end of 40 cycles to verify PCR product identity (0.2°C/sec increase from 45–95°C with continuous fluorescence readings). β-Actin primers were used as internal controls for quantification, and GAPDH primers were used to normalize samples for comparison. Primers were designed to have similar GC content and melting temperatures by using Vector NTI Suite InforMax (Bethesda, MD). In general, amplicons were between 100 and 150 nucleotides in length. GAPDH and β-actin primers were previously described (24). Primer sequences used for this analysis are available at www.sunysb.edu/biochem/MCB/Faculty/Mackow.html.

Results

DNA Array Analysis of Endothelial Cell Responses to Hantavirus Infection. Endothelial cells were infected at a MOI of 1 by pathogenic and nonpathogenic hantaviruses to compare cellular transcriptional responses regulated by HPS- or HFRS-causing hantaviruses. Each hantavirus infected >80% of endothelial cells 1 day p.i. as detected by immunostaining of the viral nucleocapsid protein within cells. We examined mRNAs from HTNV-, NY-1V-, and PHV-infected endothelial cells and compared transcriptional responses to mock-infected endothelial cell controls at early (1 day) and late (4 days) times p.i. Affymetrix chips, containing an array of ≈12,000 known human genes, were used to detect and compare changes in cellular mRNA levels directed by hantavirus infection. Affymetrix MICROARRAY ANALYSIS SUITE, Ver. 4.0.1, was used to compare viral array data to controls. Output was hierarchically presented in two gene clusters differentiated by an increase or decrease in abundance relative to controls. Changes in mRNA levels (>2-fold) induced by hantaviruses 4 days p.i. were functionally clustered and are displayed in Table 1. A complete listing of transcriptional changes at early and late times after infection is provided at www.sunysb.edu/biochem/MCB/Faculty/Mackow.html.

By comparing infected vs. mock-infected DNA arrays, transcription of 117 genes increased and 25 genes decreased in abundance >3-fold at late times after hantavirus infection. Despite the presence of similar numbers of N protein-expressing cells after HTNV, NY-1V, and PHV infection, there was a striking difference in the induction of genes 1 day p.i. between PHV and pathogenic hantaviruses. PHV altered the transcription of 67 genes 1 day p.i. (47 induced), whereas only two genes were induced by NY-1V 1 day p.i. (one gene by HTNV).

At late times p.i., a number of hantavirus-directed transcriptional responses were similar between pathogenic and nonpathogenic hantaviruses, suggesting some common cellular responses to hantaviruses or viruses in general. However, each hantavirus also induced unique transcriptional responses from endothelial cells. NY-1V and HTNV differentially regulated 13 genes (12 induced) that were not altered by infection with PHV (Table 2). Twelve genes were regulated (nine induced) by HTNV and PHV but not by NY-1V. HTNV altered the transcription of 45 genes that were unchanged after infection by NY-1V or PHV, and NY-1V regulated 10 genes that were not altered by infection with HTNV or PHV (Table 2). As a result, a fingerprint of common and unique endothelial cell responses directed by pathogenic and nonpathogenic hantaviruses has been defined by using DNA arrays.

Hantavirus-Specific Cellular Responses. At late times p.i., each hantavirus induced unique cellular transcriptional responses. Only HTNV induced IFN-β, IL-8, IL-6, GRO-β, -γ, GM-CSF, G-CSF, CKA-3, IL-7R, Cox-2, intercellular adhesion molecule (ICAM), complement component 1 inhibitor, Bcl-6, and others >3-fold (Table 2). An additional five chemokine genes were induced by both HTNV and PHV, whereas only three were directed by NY-1V. By real-time PCR, HTNV and PHV induced IL-8 36- and 6-fold, respectively, although IL-8 was not induced by NY-1V. HTNV caused the largest induction of each chemokine or cytokine gene, suggesting a fundamentally important difference in HTNV-directed endothelial cell responses.

NY-1V infection induced or repressed only 10 distinct genes from PHV or HTNV. However, 13 additional genes were altered by HTNV and PHV that are not altered by NY-1V, indicating that at least 23 genes are differentially regulated by NY-1V and other hantaviruses (Table 2). Notably lacking from NY-1V-directed responses were nearly all chemokine responses directed by HTNV (3/14). Consistent with this is the apparent lack of NF-κB-directed transcriptional responses induced by HTNV, including A20, IL-8, RANTES, E-Selectin, tumor necrosis factor receptor-associated factors (TRAFs), IAP1, and others. The down-regulation of TRAF2 by NY-1V suggests that one pathway for NF-κB activation may be regulated by HPS-associated viruses. This possibility is supported by an absence of NF-κB activation after NY-1V infection (<2-fold) using NF-κB luciferase reporters. The combination of these findings suggests that selective regulation of endothelial cell-signaling pathways during hantavirus infection may direct virus-specific transcriptional responses.

Temporal Regulation of Endothelial Cell Transcription by Hantaviruses. IFN inducible genes (28 ISGs) represent the largest category of hantavirus-directed transcriptional responses at late times p.i. ISGs included 2′, 5′-oligoadenylate synthetase (OAS genes), Mx genes, IFN regulatory factors, and the STAT2 transcription factor. However, only PHV induced 24 ISGs between 3.8- and 229-fold 1 day p.i., including MxA (161-fold) (Table 1). In all, PHV induced 21 genes >10-fold and 11 genes >20-fold 1 day p.i. NY-1V only directed 1 ISG (MxA, 3.7-fold) at early times, whereas HTNV failed to direct any early ISG cellular responses. At late times p.i., all three hantaviruses induced >23 ISGs by >5-fold, with 14 genes induced >20-fold by one or more hantaviruses.

After PHV infection, temporally induced genes were not limited to ISGs. PHV induced a total of 47 genes 1 day p.i., including RANTES, IP10, MHC I, osteopontin, TRAIL, and iNOS, while

Table 1. Fold change 4 days p.i.

Gene	Accession no.	Fold change* 4 days p.i. HTNV	NY-1V	PHV
IFN Inducible				
MxA†	M33882	198.6	195.6	212
OAS 59kDa isoform†	AJ225089	98	33	20
CIG49†	AF026939	89	55	129
HEM45†	U88964	86	26	12
IFN-inducible 56-kDa protein†	M24594	74	66	59
IFN-inducible protein 9-27†	J04164	71	66	28
CIG5†	AF026941	51	31	57
MxB†	M30818	39	21	22
1 6-Kb mRNA for OAS†	X04371	39	29	25
69-kDa OAS†	M87284	35	30	40
IFP35†	L78833	33	21	47
OAS E gene†	M11810	32	24	51
IRF 7B†	U53831	29	24	5.2
ISG-54K†	M14660	20	8.6	22
Microtubular aggregate protein p44†	D28915	18	15	15
IP-30	J03909	16	4	
IFN-induced 17-/15-kDA protein†	M13755	15	15	15
GBP2†	M55542	12	13	3.9
IRF1	L05072	12	7.3	3.4
IFN-inducible peptide (6–16)†	U22970	11	9.7	44
Nuclear phosphoprotein IFN induced†	L22342	11	11	11
ISGF-3†	M97935	10	8.3	5.3
RI58	U34605	8.1	8	16
BST-2†	D28137	6.2	5.6	5.2
p27†	X67325	5.9	5.9	9.3
IRF2	X15949			7.6
Staf50†	X82200	4.2	3.5	2.2
Nmi†	U32849	3.8	3.2	2
Chemokines/cytokines				
I-TAC†	AF030514	41	27	13
IP10†	X02530	24	8.3	4.4
GRO-β	M36820	18		2.1
RANTES†	M21121	17		16
IL8	M28130	15		
GM-CSF	M13207	16		
G-CSF	X03656	11		
CKA-3	U81234	13		
Chemokine exodus-1	U64197	12		4.2
CX3C chemokine precursor†	U84487	9.5	3.2	2
GRO-α	X54489	9.1		3.2
GRO-γ	M36821	5.2		
IL-6	X04430	4.7		
IFN-β 1	V00535	3.6		
Apoptosis				
XAF1	X99699	7.6	5.5	15
TRAIL†	U37518	5.2	5.4	
Inhibitor of apoptosis protein 1	U45878	4		4.7
IL-1-β convertase (IL1BCE)	M87507	6.9	4.7	3.4
Immune response				
MHC, class I, F†	AL022723	41	15	34
Complement factor B	L15702	8.6		11
Complement component C1R	J04080	7		3.1
Complement component 1 inhibitor	X54486	4.3		
HLA class I locus C heavy chain†	X58536	4.1	3.3	3.1
ATL-derived PMA-responsive peptide	D90070	4		
Lymphocyte antigen (HLA-G1)	M90683	3.3		2.4
BTF4	U90546	3.3		
52-kD SS-A/Ro autoantigen†	M62800	4.5	3.5	2.6
RING4†	X57522	8.9	6	7.3
K12 protein precursor†	U77643	13	9.8	12
Cell surface receptors				
Mac-2 binding protein	L13210	34	4.5	13
uPA receptor	X74039	1.7		15
VCAM1	M30257	12	3.1	
LY-6-related protein (9804)†	U66711	9.7	6.8	27
VEGFR 165/neuropilin (VEGFR165)	AF016050			8.8
E-Selectin	M24736	8.1		1.9
Galectin-9 isoform†	AB006782	7.5	5.4	4
ICAM-1	M24283	6.6		2.2
IL-7 receptor	AF043129	6		
IFNAR2 (IFN Receptor)	L42243	4.3		
DARC (Duffy antigen/chemokine receptor)	X85785	4		
Integrin variant β4E (ITGB4)	AF011375			5
Transporters/channels				
Low-affinity sodium glucose cotransporter	AL008723			8.0
Glutamate transporter	U08989			4.2
Potassium channel (HPCN1)	M55513		3.2	
β-1,4-glactosyltransferase	AB004550			5.0
Signaling				
Dual-specificity protein phosphatase	U15932	4.4		3.1
Protein-tyrosine phosphatase	D11327		3.4	
Serine/threonine protein kinase SAK	Y13115			4.5
JNK activating kinase (JNKK1)	U17743			3.2
cAMP-dependent protein kinase cat. subunit	X07767		3.2	
Calcium/calmodulin-depend. protein kinase I	U66063			9.4
TRAF1	U19261	5.2		
TNF-α-inducible protein A20	M59465	7.6		2.9
B94 protein=TNF-α IP2	M92357	4.8		
MyD88	U70451	2.7	3.1	
Orphan G-protein-coupled receptor (RDC1)	U67784	4.4	3.3	
Calcineurin A2	M29551			−19
TRAF2	U12597		−6.0	
Protein tyrosine phosphatase δ	L38929			−5.6
InsP3,5-phosphatase	X77567	−3.7	−3.5	
MAP kinase kinase MEK5b	U71087	−3.1		
Orphan G-protein-coupled receptor	L06797	−3.7		

*Values are fold are fold changes. Fold repression is indicated by a minus sign.
†Genes induced day 1 by PHV.

repressing another 20 genes including SOS, ras interactor, rap2, SNARE, decoy receptor 2, and the CDK inhibitor KIP2. Only three genes were repressed by NY-1V or HTNV (3- to 4-fold) 1 day p.i., whereas one gene, osteopontin, is induced to similar levels by all three viruses (11- to 12-fold). These findings demonstrate a dramatic temporal difference in endothelial cell transcriptional responses between pathogenic and nonpathogenic hantaviruses.

Analysis of Selected Genes. We have used quantitative real-time PCR to examine the transcriptional activation of 14 selected genes that were differentially regulated by DNA microarray analysis. Real-time PCR values were normalized to GAPDH mRNA levels, and DNA array changes verified by quantitative real-time PCR are presented in Table 3. Similar transcriptional changes were identified by real-time PCR and by DNA array analysis, although larger changes were generally observed by using real-time PCR. HTNV induced MxA 559-fold, MxB 160-fold, IL-8 34-fold, RANTES 64-fold, IFP35 96-fold, Cox2 21-fold, GRO-β 22-fold, ICAM-1 16-fold, and vascular cell adhesion molecule 1 (VCAM-1) 44-fold. The increased sensitivity of real-time PCR as well as the potential for chip saturation at high mRNA induction levels accounts for

Table 2. Hantavirus-specific changes

Gene	Accession no.	>3-Fold changes HTNV	NY-1V	PHV
Regulated by NY-1V only				
Protein-tyrosine phosphatase	D11327		3.4	
Keratin 2e (KRT2E)	AF019084		3.3	
cAMP-dependent PK catalytic subunit	X07767		3.2	
Potassium channel (HPCN1)	M55513		3.2	
RAD52	U27516		3	
Aldolase B	X02747		3.0	
TRAF2	U12597		−6	
Tat interactive protein (TIP60)	U74667		−4	
Galactokinase (GALK1)	L76927		−4.4	
MyD88	U70451		3.1	
Regulated by HTNV only				
GRO-β	M36820	18		
GM-CSF	M13207	16		
IL8	M28130	15		
Chemokine α 3 (CKA-3)	U81234	13		
Mn-superoxide dismutase	X07834	12		
G-CSF	X03656	11		
E-Selectin	M24736	8.1		
TNF-α inducible protein A20	M59465	7.6		
ICAM-1	M24283	6.6		
IL-7 Receptor	AF043129	6		
NF-IL6-β protein	M83667	5.5		
Cox-2	U04636	5.3		
GRO-γ	M36821	5.2		
TRAF1	U19261	5.2		
B94 protein	M92357	4.8		
IL6	X04430	4.7		
Complement component 1 inhibitor	X54486	4.3		
Histone H2A.2	L19779	4.3		
IFNAR2 (interferon receptor)	L42243	4.3		
Stat2	U18671	4		
ATL-derived PMA-responsive peptide	D90070	4		
DARC (Duffy antigen/chemokine recept)	X85785	4		
TSC403 protein	AB013924	4		
Cholesterol 25-hydroxylase	AF059214	3.8		
PDGFR β-like tumor sup. (PRLTS)	D37965	3.7		
Jun-B	X51345	3.6		
IFN β 1	V00535	3.6		
Stanniocalcin precursor (STC)	U25997	3.6		
Heat shock transcription factor 4	D87673	3.4		
Nuclear autoantigen (SP-100)	M60618	3.4		
My1 (PML)	X63131	3.3		
Bcl-6	U00115	3.3		
Butyrophilin (BTF4)	U90546	3.3		
Lymphocyte antigen (HLA-G1)	M90683	3.3		
Glutathione S-transferase subunit 4	X08020	−7.8		
SNARE protein Ykt6	U95735	−3.8		
Orphan G-protein-coupled receptor	L06797	−3.7		
Pig3 p53 induced protein	AF010309	−3.5		
Iron regulatory factor	Z11559	−3.4		
Cartilage-associated protein (CASP)	AJ006470	−3		
AQP-1	U41518	−3.3		
Ubiquitin-fusion degradation protein 2	AF043117	−3.3		
MAP kinase kinase MEK5b	U71087	−3.1		
Pyruvate dehydrogenase kinase 2 (PDK2)	L42451	−3		
Dr1-associated corepressor (DRAP1)	U41843	−3		
Regulated by HTNV + PHV but not NY-1				
RANTES	M21121	17		16
Chemokine exodus-1	U64197	12		4.2
GRO-α	X54489	9.1		3.2
PDECGF	M63193	14		26
Insulin-like growth factor bind. protein 6	M62402	3.9		4.1
Complement factor B	L15702	8.6		11
Complement component C1R	J04080	7		3.1
Glutathione S-transferase s4	M16594	−7.8		−4.5
Inhibitor of apoptosis protein 1	U45878	4		4.7
Dual-specificity protein phosphatase	U15932	4.4		3.1
Initiation factor 4B	X55733	−4.9		−3.5
Connexin 37	M96789	−4.4		−3.2
Decoy receptor 2	AF029761	−3.3		−2.5
Regulated by HTNV + NY-1V but not PHV				
IP-30	J03909	16	4	
VCAM1	M30257	12	3.1	
CX3C chemokine precursor	U84487	9.5	3.2	
RING4	X57522	8.9	6	
Deoxyribonuclease III (drn3)	AJ243797	7.1	3.9	
Apolipoprotein L	AF019225	5.5	4	
TRAIL	U37518	5.2	5.4	
52-kD SS-A/Ro autoantigen	M62800	4.5	3.5	
SP 100-B	U36501	4.9	4.7	
Staf50	X82200	4.2	3.5	
Nmi	U32849	3.8	3.2	
Orphan G-protein-coupled receptor	U67784	4.4	3.3	
InsP3,5-phosphatase	X77567	−3.7	−3.5	
Regulated by PHV only				
α-tubulin	X06956			27
uPA receptor	X74039			15
Calcium/calmod.-depend. protein kinase II	U66063			9.4
Low affinity sodium glucose cotransporter	AL008723			8.0
IRF2	X15949			7.6
Integrin variant β4E (ITGB4)	AF011375			5
β-1,4-galactosyltransferase	AB004550			5
Serine/threonine protein kinase SAK	Y13115			4.5
Glutamate transporter	U08989			4.2
Cdc6-related protein (HsCDC6)	U77949			4.1
β-thromboglobulin-like protein	M17017			3.9
Microtubule-associated protein 1B	L06237			3.9
NF-AT3	L41066			3.4
Laminin-related protein (LamA3)	L34155			3.2
JNK activating kinase (JNKK1)	U17743			3.2
Collagen	D21337			3.1
Cdc25A	M81933			3.1
Calcineurin A2	M29551			−19
Protein tyrosine phosphatase δ	L38929			−5.6
Collagen α-2 type I	J03464			−4.7
p300	U01877			−4.2
Matrilin-2 precursor	U69263			−3.7
High mobility group protein (HMG2a)	Y10043			−3.4
n-myc	Y00664			−3.3

*Fold repression is indicated by a minus sign.

differences in the magnitude of the induction detected by the two approaches. This possibility accounts for differences in IL-8 detected by real-time PCR for PHV but absent in the array. Similarly, VCAM was not induced by PHV as analyzed by the DNA array, although real-time PCR (18-fold) and Western blot (not shown) indicate that VCAM was induced by PHV. In contrast, the low-level induction of VCAM by NY-1V was not verified by Western blot, although this may be the result of the low-level RNA increase detected and the relatively insensitive nature of Western blots relative to PCR.

Discussion

Viruses engage and regulate cellular signaling pathways and elicit specific transcriptional responses to successfully infect cells. The strategy that hantaviruses use during infection of their natural animal hosts fails within humans and results in two types of disease (1). However, both pathogenic and nonpathogenic hantaviruses infect human endothelial cells, suggesting that basic differences in virus–cell interactions may contribute to disease (2). We have used DNA arrays to define endothelial cell responses elicited by hantaviruses that cause HPS or HFRS or that are not associated with any human disease. These studies have revealed a central temporal difference in cellular responses elicited by pathogenic and nonpathogenic hantaviruses as well as

Table 3. Real-time PCR analysis

Gene	Fold induction vs. control		
	HTNV	NY-1V	PHV
MxA	559	552	475
MxB	160	64	163
GBP2	6.8	6.5	10.2
IFN-inducible protein 9-27	6.7	3.6	10.5
IL-8	34	1	6.3
RANTES	64	2	6
MHC-I	13	24	279
IFP35	96	80	57
GTP-cyclohydrolase	5.3	4.5	6.5
Cox2	21	3.1	4.3
GRO-β	22	1.3	50
XAF1	8.5	3.8	54
ICAM-1	16	4.2	32
VCAM-1	44	4.3	18

the induction of unique genes by HPS- and HFRS-causing hantaviruses.

Approximately five times more genes are up-regulated than down-regulated after hantavirus infection. This finding is in stark contrast to transcriptional responses after infection by influenza virus, where 329 genes were down-regulated and only 39 genes were up-regulated (25). Influenza virus is also a segmented negative-stranded RNA virus, and these disparate findings suggest that even viruses that replicate similarly use dramatically different strategies to regulate cellular transcriptional responses.

Responses to Pathogenic and Nonpathogenic Hantaviruses. The most striking difference in hantavirus-induced cellular transcription is the dramatic induction of endothelial cell responses 1 day p.i. by only the nonpathogenic hantavirus PHV. PHV altered gene expression of 67 genes 1 day p.i., as compared with the regulation of three or less genes by HPS- or HFRS-causing hantaviruses. MxA has known antiviral effects and is induced 161-fold by PHV 1 day p.i. compared with 3.7-fold by NY-1V and <2-fold by HTNV (26). Because 24 IFN-specific genes were induced to high levels (3.8- to 229-fold) by PHV early on, it is possible that endothelial cell responses may limit the replication of nonpathogenic hantaviruses shortly after infection. This possibility is consistent with differential viral titers observed after infection of endothelial cells by pathogenic and nonpathogenic hantaviruses. At late times, MxA is induced about 200-fold by all three hantaviruses, along with a variety of additional IFN responses. Similar to influenza virus, the rapid induction of responses by PHV may contribute to rapid viral clearance, decreased replication, and the lack of disease by PHV (27). In contrast, late onset of HTNV-directed IFN and chemokine responses may contribute to an inflammatory immune-mediated disease process associated with HTNV (6). These findings suggest that pathogenic hantaviruses may delay or regulate the onset of endothelial cell responses that limit replication.

Pretreatment of cells with IFN-β is reported to inhibit HTNV replication, and IFN-β mRNA was induced 23-fold by HTNV 3 days p.i (3, 28). This response is verified by our DNA array data, which also confirm the lack of IFN response 1 day p.i. with HTNV. The low 3.6-fold induction of IFN-β detected by DNA arrays may reflect differences in the MOI used between the two experiments (0.1 vs. 1) (3). Paracrine effects of IFN after infection at low MOIs could account for differences in the levels of IFN-β induction and explain why antibodies to IFN-β enhanced HTNV replication when only 10% of cells were initially infected. The autocrine induction of ISGs after synchronous hantavirus infection is likely to account for the majority of the IFN response (29). The differential induction of IFN-β by HTNV and the early induction of ISGs by PHV suggest that hantaviruses may selectively direct distinct IFN responses during infection (26).

Induction of Complement Components by HTNV. A role for complement in immune-mediated complications of HFRS disease has been reported (8, 9). Complement components were induced by HTNV and PHV infection, although only HTNV induced complement component 1 inhibitor. Complement components interact in a highly regulated cascade by binding to specific complement receptors on cells and trigger the activation of immune responses. The deposition and clearance of immune complexes from the circulation may actively contribute to aspects of HFRS (8, 9). Although PHV may similarly induce complement components, it is unclear whether the early induction of ISGs by nonpathogenic hantaviruses makes later complement induction irrelevant to disease, because PHV is unable to replicate and spread. In contrast, the ability of HTNV to induce complement factor B and complement component C1r ties endothelial cell transcriptional events to immune complex contributions by HFRS causing hantaviruses.

Induction of Proinflammatory Responses. All three viruses induced endothelial cell transcription of T cell α chemoattractant and IP10. Interestingly, DNA arrays indicate that GRO-α, RANTES, and chemokine exodus-1 are induced by both HTNV and PHV infection but not by NY-1V. The induction of IP10 and RANTES by HTNV virus was previously reported and confirmed by our DNA array findings (5). HTNV infection resulted in a 2- to 3-fold increase in secreted RANTES. Using ribonuclease protection assays, RANTES was also reported to be induced by Sin Nombre virus (SNV, HPS associated), although RANTES secretion was not presented (5). We cannot exclude the possibility that SNV and NY-1V have different effects on RANTES induction. However, our DNA array data suggest that RANTES is induced by HTNV but not by NY-1V, and these findings were verified by real-time PCR in which HTNV induced a 32-fold increase in RANTES mRNA over NY-1V (Table 3). Our results agree with a separate report that IL-1 is not induced by HTNV but disagree with a reported lack of IL-6 induction by HTNV detected by reverse transcription–PCR (3). However, previously reported responses used a low initial MOI and were analyzed 1–3 days after HTNV infection (3). As a result, these findings are consistent with our negative IL-6 induction data during early stages of infection.

HTNV induces a fundamentally different proinflammatory chemokine response from infected endothelial cells than other hantaviruses. At least 12 chemokine genes were induced >9-fold by HTNV, and HTNV also induced nine immune cell-recruiting receptors and a variety of immune response-related genes. Only HTNV induced Cox2, NF-IL6, GM-CSF, and G-CSF genes, which stimulate cellular inflammatory responses or hematopoiesis (22). GRO-γ, CKA-3, IL-8, and IL-6 were also induced by HTNV, but not NY-1V, and are strong neutrophil or lymphocyte chemoattractants (20, 21, 30). Cytokines and chemokines can alter vascular integrity or modify thrombotic potential and thus may contribute to pathogenesis (21, 31, 32). The increased chemokine response and the magnitude of these responses to HTNV may also contribute to aspects of HFRS that are different from HPS.

ICAM-1, E-Selectin, and VCAM-1 were induced by HTNV, and these cell adhesion molecules are central to leukocyte adherence and the propagation of inflammatory responses (19). DNA arrays and real-time PCR show that HTNV induces VCAM, ICAM, and E-Selectin, and two of these responses were not observed after infection with NY-1V. These findings are in contrast to a report indicating no change in these receptors between mock- and hantavirus-infected cells by ribonuclease protection assays (5). DNA array and real-time PCR data suggest that HTNV elicits a unique set of immune cell recruiting and attachment responses including VCAM, ICAM, and E-Selectin. Western blot analysis of infected endothelial cells confirmed increases in VCAM and

ICAM receptors in response to HTNV. Because HFRS has been described as at least a partially immune-mediated disease, endothelial cell responses involved in immune cell recruitment may play specific roles in HTNV-directed pathogenesis.

NY-1V-Specific Endothelial Cell Responses. Each hantavirus elicited unique transcriptional responses from infected endothelial cells. Along with differences in chemokine induction by hantaviruses, NY-1V infection uniquely down-regulated TRAF2 and up-regulated the transcription of a potassium channel. Interestingly, $\alpha v\beta 3$ integrins used by pathogenic hantaviruses are linked to potassium channel activity, and potassium channels have been shown to act as vasodilators in some cells (33, 34). These findings suggest the potential importance of potassium channel induction by NY-1V in vascular disease.

Transcriptional responses observed after infection are likely to reflect pathway-specific signaling responses activated or regulated by hantaviruses. NY-1V represses TRAF2 gene expression and TRAF2 mediates NF-κB activation from a number of upstream signals (35, 36). NF-κB inducible genes like IL-8, GRO genes, RANTES, GM-CSF, G-CSF, E-Selectin, ICAM, and Cox2 were not induced by NY-1V but were induced by HTNV (37). Consistent with this, we have also found that NY-1V does not activate NF-κB during infection. These findings suggest that NY-1V may prevent or selectively down-regulate NF-κB-directed responses that contribute to chemokine induction and immune cell recruitment (37). The means by which HTNV and NY-1V differentially regulate NF-κB and NF-κB-directed cellular responses require further investigation but may represent an underlying difference between NY-1V and HTNV that contributes to specific disease manifestations.

PHV Induces Vascular Endothelial Cell Growth Factor Receptor (VEGFR) and Urokinase Plasminogen Activator Receptor (UPAR). We have previously shown that only pathogenic hantaviruses use $\alpha v\beta 3$ integrins for cellular entry (11, 12). The interaction of $\alpha v\beta 3$ with VEGFR-2 was recently reported and links vascular permeability effects of $\beta 3$ and VEGF (formerly called vascular permeability factor) to receptors regulated by pathogenic hantaviruses (15, 16, 38, 39). We found that PHV up-regulated the VEGFR-165, neuropilin-1. Because this receptor modulates VEGF responses, up-regulation of this gene by PHV may ameliorate permeabilizing effects of VEGF during infection by nonpathogenic hantaviruses (40–44). However, there are multiple VEGF receptors and coreceptors that could impact endothelial responses.

PHV also induced the urokinase plasminogen activator receptor (UPAR), which, like $\alpha v\beta 3$, is a receptor for vitronectin (45, 46). Altered UPAR induction could dynamically regulate endothelial cell responses to plasminogen activator or vitronectin during infection and regulate $\beta 3$ integrin-directed VEGF responses, which may be dysregulated by pathogenic hantaviruses. These findings suggest two potential means for altering vascular permeability that are differentially regulated by pathogenic and nonpathogenic hantaviruses.

Although we have defined endothelial cell transcriptional responses, our findings are also limited by their intended focus on the endothelium. These findings exclude responses from immune cells and complex immune cell–endothelial cell interactions that are also likely to contribute to multifactorial causes of hantavirus disease. However, in focusing on the primary site of hantavirus infection, we have addressed key vascular responses and identified many factors and receptors that regulate immune cell responses and endothelial cell barrier functions central to hantavirus diseases.

The potential role of endothelial cell responses in hantavirus pathogenesis remains speculative, but these connections are vital for defining factors that contribute to hantavirus disease. The ability to identify cellular genes whose functions provide potential mechanistic roles in the infectious disease process underscores the utility of gene array technology in the study of viral pathogenesis. The global analysis of changes in endothelial cell mRNA levels demonstrates dramatic temporal changes between pathogenic and nonpathogenic hantaviruses as well as selected genes that are induced by hantaviruses that cause HPS or HFRS. These findings direct our attention to cellular responses that are likely to contribute to HPS and HFRS diseases and provide potential points for disease intervention.

We thank Karen Endriss, Yu Son Chong, and Joanne Mackow for technical assistance. This work was supported by a Merit Award from the Veterans Administration, and by National Institutes of Health Grants AI047873 and AI044917.

1. Schmaljohn, C. & Hjelle, B. (1997) *Emerg. Infect. Dis.* **3,** 95–104.
2. Yanagihara, R. & Silverman, D. J. (1990) *Arch. Virol.* **111,** 281–286.
3. Pensiero, M. N., Sharefkin, J. B., Dieffenbach, C. W. & Hay, J. (1992) *J. Virol.* **66,** 5929–5936.
4. Zaki, S., Greer, P., Coffield, L., Goldsmith, C., Nolte, K., Foucar, K., Feddersen, R., Zumwalt, R., Miller, G., Rollin, P., Ksiazek, T., Nichol, S. & Peters, C. (1995) *Am. J. Pathol.* **146,** 552–579.
5. Sundstrom, J. B., McMullan, L. K., Spiropoulou, C. F., Hooper, W. C., Ansari, A. A., Peters, C. J. & Rollin, P. E. (2001) *J. Virol.* **75,** 6070–6085.
6. Lee, H. W. (1982) *Scand. J. Infect. Dis. Suppl.* **36,** 82–85.
7. Nolte, K. B., Feddersen, R. M., Foucar, K., Zaki, S. R., Koster, F. T., Madar, D., Merlin, T. L., McFeeley, P. J., Umland, E. T. & Zumwalt, R. E. (1995) *Hum. Pathol.* **26,** 110–120.
8. Vapalahti, O., Lundkvist, A. & Vaheri, A. (2001) *Curr. Top. Microbiol. Immunol.* **256,** 153–169.
9. Gavrilovskaya, I., Podgorodnichenko, V., Apekina, N., Gordbachkova, E., Bogdanova, S., Kodkind, G., Linev, M. & Chumakoc, M. (1987) *Microbiol. J.* **49,** 71–76.
10. Paakkala, A., Mustonen, J., Viander, M., Huhtala, H. & Pasternack, A. (2000) *Clin. Nephrol.* **53,** 424–431.
11. Mackow, E. R. & Gavrilovskaya, I. N. (2001) *Curr. Top. Microbiol. Immunol.* **256,** 91–115.
12. Gavrilovskaya, I. N., Shepley, M., Shaw, R., Ginsberg, M. H. & Mackow, E. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7074–7079.
13. Gavrilovskaya, I. N., Brown, E. J., Ginsberg, M. H. & Mackow, E. R. (1999) *J. Virol.* **73,** 3951–3959.
14. Hodivala-Dilke, K. M., McHugh, K. P., Tsakiris, D. A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, F. P., Coller, B. S., Teitelbaum, S. & Hynes, R. O. (1999) *J. Clin. Invest.* **103,** 229–238.
15. Reynolds, L. E., Wyder, L., Lively, J. C., Taverna, D., Robinson, S. D., Huang, X., Sheppard, D., Hynes, R. O. & Hodivala-Dilke, K. M. (2002) *Nat. Med.* **8,** 27–34.
16. Dvorak, H. F., Brown, L. F., Detmar, M. & Dvorak, A. M. (1995) *Am. J. Pathol.* **146,** 1029–1039.
17. Kevil, C. G., Payne, D. K., Mire, E. & Alexander, J. S. (1998) *J. Biol. Chem.* **273,** 15099–15103.
18. Mogford, J. E., Davis, G. E., Platts, S. H. & Meininger, G. A. (1996) *Circ. Res.* **79,** 821–826.
19. Aplin, A. E., Howe, A., Alahari, S. K. & Juliano, R. L. (1998) *Pharmacol. Rev.* **50,** 197–263.
20. Gerszten, R. E., Garcia-Zepeda, E. A., Lim, Y. C., Yoshida, M., Ding, H. A., Gimbrone, M. A., Jr., Luster, A. D., Luscinskas, F. W. & Rosenzweig, A. (1999) *Nature* **398,** 718–723.
21. Mackay, C. R. (2001) *Nat. Immunol.* **2,** 95–101.
22. Broxmeyer, H. E., Sherry, B., Cooper, S., Lu, L., Maze, R., Beckmann, M. P., Cerami, A. & Ralph, P. (1993) *J. Immunol.* **150,** 3448–3458.
23. Gavrilovskaya, I., LaMonica, R., Fay, M. E., Hjelle, B., Schmaljohn, C., Shaw, R. & Mackow, E. R. (1999) *J. Clin. Microbiol.* **37,** 122–126.
24. Medhurst, A. D., Harrison, D. C., Read, S. J., Campbell, C. A., Robbins, M. J. & Pangalos, M. N. (2000) *J. Neurosci. Methods* **98,** 9–20.
25. Geiss, G. K., An, M. C., Bumgarner, R. E., Hammersmark, E., Cunningham, D. & Katze, M. G. (2001) *J. Virol.* **75,** 4321–4331.
26. Samuel, C. E. (2001) *Clin. Microbiol. Rev.* **14,** 778–809.
27. Garcia-Sastre, A., Durbin, R. K., Zheng, H., Palese, P., Gertner, R., Levy, D. E. & Durbin, J. E. (1998) *J. Virol.* **72,** 8550–8558.
28. Tamura, M., Asada, H., Kondo, K., Takahashi, M. & Yamanishi, K. (1987) *Antiviral Res.* **8,** 171–178.
29. Der, S. D., Zhou, A., Williams, B. R. & Silverman, R. H. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 15623–15628.
30. Luster, A. D. (1998) *N. Engl. J. Med.* **338,** 436–445.
31. Maruo, N., Morita, I., Shirao, M. & Murota, S. (1992) *Endocrinology* **131,** 710–714.
32. Mahalingam, S. & Karupiah, G. (1999) *Immunol. Cell Biol.* **77,** 469–475.
33. Sobey, C. G. (2001) *Arterioscler. Thromb. Vasc. Biol.* **21,** 28–38.
34. Platts, S. H., Mogford, J. E., Davis, M. J. & Meininger, G. A. (1998) *Am. J. Physiol.* **275,** H1449–H1454.
35. Inoue, J., Ishida, T., Tsukamoto, N., Kobayashi, N., Naito, A., Azuma, S. & Yamamoto, T. (2000) *Exp. Cell Res.* **254,** 14–24.
36. Bradley, J. R. & Pober, J. S. (2001) *Oncogene* **20,** 6482–6491.
37. Denk, A., Goebeler, M., Schmid, S., Berberich, I., Ritz, O., Lindemann, D., Ludwig, S. & Wirth, T. (2001) *J. Biol. Chem.* **276,** 28451–28458.
38. Byzova, V. T., Goldman, K. C., Pampori, N., Thomas, A. K., Bett, A., Shattil, J. S. & Plow, F. E. (2000) *Mol. Cell* **6,** 851–860.
39. Soldi, R., Mitola, S., Strasly, M., Defilippi, P., Tarone, G. & Bussolino, F. (1999) *EMBO J.* **18,** 882–892.
40. Soker, S., Takashima, S., Miao, H. Q., Neufeld, G. & Klagsbrun, M. (1998) *Cell* **92,** 735–745.
41. Neufeld, G., Cohen, T., Gengrinovitch, S. & Poltorak, Z. (1999) *FASEB J.* **13,** 9–22.
42. Verheul, H., Jorna, A., Hoekman, K., Broxterman, H., Martijn, F., Gebbink, B. & Pinedo, H. (2000) *Blood* **96,** 4216–4221.
43. Hiratsuka, S., Maru, Y., Okada, A., Seiki, M., Noda, T. & Shibuya, M. (2001) *Cancer Res.* **61,** 1207–1213.
44. Robinson, C. & Stringer, S. (2001) *J. Cell Sci.* **114,** 853–865.
45. Loskutoff, D. J., Curriden, S. A., Hu, G. & Deng, G. (1999) *APMIS* **107,** 54–61.
46. Kanse, S. M., Kost, C., Wilhelm, O. G., Andreasen, P. A. & Preissner, K. T. (1996) *Exp. Cell Res.* **224,** 344–353.