Effect of Immunosuppression on Gene Expression in the HSV-1 Latently Infected Mouse Trigeminal Ganglion

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PURPOSE. To determine alterations in expression of genes in herpes simplex virus (HSV)-1 latently infected mouse trigeminal ganglia (TGs), after treatment with cyclophosphamide and dexamethasone.

METHODS. Scarified corneas of female BALB/c mice were inoculated with HSV-1 strain McKrae. Four weeks after inoculation, cyclophosphamide and dexamethasone were intravenously injected to induce HSV-1 reactivation. Uninfected mice were also treated with the immunosuppressants. Four groups of animals were studied: uninfected, not treated; uninfected, drug treated; latently infected, not treated; and latently infected, drug treated. Poly(A)+ mRNA from the TGs of each group was reverse transcribed, labeled with 32P, incubated on a 1185-gene array, and analyzed by phosphorimaging. As a comparison and to confirm microarray results, semiquantitative RT-PCR was also performed for selected genes.

RESULTS. The immunosuppressive drugs significantly increased expression of two genes (calpain 1 light chain and guanine nucleotide-binding protein α-stimulating polypeptide [GNAS]) in the ganglia of uninfected mice compared with those in untreated uninfected mice. Ten genes were shown to be significantly increased in the latent TGs of mice treated with immunosuppressants compared with latently infected untreated mice. These genes were prostaglandin E2 receptor EP4 subtype (PTGER4), insulin promoter factor 1 (IPF1), glutathione S-transferase μ2, cyclin D2, peripherin, plasma glutathione peroxidase, methyl CpG-binding protein 2, retinal S-antigen, ErbB2 proto-oncogene, and GNA5. Eight genes were shown to be significantly decreased in the HSV-1 latent TGs treated with the drugs, compared with untreated latently infected mice. These genes were peripheral myelin protein 22, decorin, transcription factor AP-1, dystroglycan 1, myelin protein zero, mitogen-activated protein kinase 3, prothymosin beta 4, and brain lipid-binding protein. The results obtained by semiquantitative RT-PCR were similar to those obtained by microarray analysis.

CONCLUSIONS. Those genes with expression altered by immunosuppressive drug treatment may play an important role in ocular HSV-1 recurrence. Changes in expression of genes in the prostaglandin pathway, a transcription factor, and an enzyme in the cell cycle are considered especially important in HSV-1 reactivation by immunosuppression and are reviewed.

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cellular mRNAs expressed in latently infected TGs that differ from the mRNAs expressed in latently infected TGs not exposed to the drugs.

We report that the transcription of a large number of genes was altered by treatment with immunosuppressive drugs. Using an array membrane containing the probes for 1185 known genes, including nine housekeeping genes, altered expression of at least 18 genes was noted in the ganglia of latently infected drug-treated mice. Possibly some of these genes with expression altered by treatment of latent disease with immunosuppressive drugs are key in the genetic mechanism of HSV-1 reactivation.

MATERIALS AND METHODS

Mouse Eye Model of Immunosuppression

Five- to 7-week-old female BALB/c mice (National Cancer Institute, Bethesda, MD) were housed in the Louisiana State University Health Sciences Center animal facility and maintained on laboratory chow and water ad libitum. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with ketamine hydrochloride (1 mg/kg; Phoenix Scientific, Inc., St. Joseph, MO), and xylazine (0.5 mg/kg; Taylor Pharmaceuticals, Decatur, IL). The corneas were scarified and inoculated with 3 μL of a viral suspension containing 2.5 × 10^7 plaque-forming units of HSV-1 strain McKrae. To verify corneal infection, slit lamp examinations and ocular swabs were performed on days 3 and 4 after inoculation.

Four weeks after inoculation, when the corneas healed, the mice were given an intravenous (IV) injection of 5 mg cyclophosphamide (Sigma; St. Louis, MO) in 0.2 mL of water, and 24 hours later an IV injection of 0.2 mg dexamethasone (Sigma) in 0.2 mL of water.29–31 Uninfected mice were also given drugs on the same schedule. One hour after the dexamethasone injection, the mice were killed, and the TGs were removed. Four groups of animals were studied: uninfected, not treated; uninfected, drug treated; latently infected, not treated; and latently infected, drug treated. Twenty-two mice (44 TGs) were examined in each group.

Isolation of PolyA+ mRNA and cDNA Array Hybridization

Total RNA was isolated from the TGs in extraction reagent (TRizol; Gibco BRL, Rockville, MD) and DNase, as part of a commercial pure total RNA labeling protocol (Atlas; Clontech). The RNA was spectrophotometrically scanned, and all samples had an absorbance ratio (A260/A280) of 2.0 or more. No significant differences in the spectral purity, rate of degradation, and yield were noted among the groups. The polyA+ mRNA fraction was obtained using biotinylated oligo (dt) and streptavidin-coated paramagnetic beads. PolyA+ mRNA was reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase in the presence of [32P]-dATP (10 μCi/μL; 3000 Ci/mmol; 35 μCi/μL; Amersham, Piscataway, NJ). cDNAs were purified in a nucleospin column and hybridized onto cDNA array panels (Atlas Mouse 1.2 Array; Clontech) with hybridization solution (ExpressHyb; Clontech). cDNA array nylon membranes were washed and exposed to autoradiographs according to the manufacturer’s protocol (PT 3140-1; Clontech). The mouse array has 1185 genes; the complete list can be found at http://www.clontech.com/atlas/genelists/index.shtml. There was approximately 1 × 10^7 cpm associated with each cDNA that was hybridized to an array membrane. For each cDNA preparation, two determinations were conducted.

Table 1. Changes in Gene Transcription in Mouse TGs after Immunosuppression with Dexamethasone and Cyclophosphamide

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession Number</th>
<th>Uninfected Trigeminal Ganglia</th>
<th>HSV-Latent Trigeminal Ganglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes showing increased transcription</td>
<td>GenBank Accession Number</td>
<td>Intensity</td>
<td>Intensity</td>
</tr>
<tr>
<td>Calpain 1 light chain</td>
<td>M16465</td>
<td>14157.5 ± 8437.9</td>
<td>20944.5 ± 11096.6</td>
</tr>
<tr>
<td>GNAS</td>
<td>Y00705</td>
<td>8317.0 ± 5762.9</td>
<td>16091.5 ± 6491.9</td>
</tr>
<tr>
<td>Glutathione S-transferase μ2</td>
<td>D13458</td>
<td>98.5 ± 12.0</td>
<td>2476.5 ± 122.3</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>X74342</td>
<td>460.0 ± 7.1</td>
<td>2979.0 ± 314.0</td>
</tr>
<tr>
<td>Peripherin</td>
<td>X15475</td>
<td>1259.5 ± 0.7</td>
<td>2012.5 ± 112.4</td>
</tr>
<tr>
<td>Plasma glutathione peroxidase</td>
<td>U13705</td>
<td>1915.5 ± 94.0</td>
<td>2676.0 ± 186.7</td>
</tr>
<tr>
<td>Methyl C6 binding protein 2</td>
<td>A072251</td>
<td>868.0 ± 128.7</td>
<td>1134.5 ± 157.7</td>
</tr>
<tr>
<td>Retinal S-antigen</td>
<td>L47239</td>
<td>713.5 ± 6.4</td>
<td>1137.5 ± 40.0</td>
</tr>
<tr>
<td>ErbB2 proto-oncogene</td>
<td>M24086</td>
<td>1281.0 ± 206.5</td>
<td>1904.5 ± 53.0</td>
</tr>
<tr>
<td>GNAS</td>
<td>Y00705</td>
<td>4195.0 ± 130.1</td>
<td>4873.0 ± 479.4</td>
</tr>
<tr>
<td>Peripherin myelin protein 22</td>
<td>M32240</td>
<td>1669.5 ± 78.5</td>
<td>747.0 ± 328.1</td>
</tr>
<tr>
<td>Decorin</td>
<td>X53029</td>
<td>2975.0 ± 219.2</td>
<td>1435.0 ± 91.9</td>
</tr>
<tr>
<td>Transcription factor AP-1</td>
<td>J04115</td>
<td>2890.0 ± 220.6</td>
<td>1588.0 ± 476.6</td>
</tr>
<tr>
<td>Dystroglycan 1</td>
<td>U45152</td>
<td>1167.5 ± 0.7</td>
<td>610.5 ± 280.7</td>
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<tr>
<td>Myelin protein zero</td>
<td>M62860</td>
<td>1938.5 ± 27.6</td>
<td>1340.5 ± 98.3</td>
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<tr>
<td>Mitogen-activated protein kinase</td>
<td>M61777</td>
<td>1518.0 ± 82.0</td>
<td>907.5 ± 456.1</td>
</tr>
<tr>
<td>Prothrombin beta 4</td>
<td>X16055</td>
<td>3834.5 ± 129.4</td>
<td>3513.0 ± 383.3</td>
</tr>
<tr>
<td>Brain lipid-binding protein</td>
<td>S69799</td>
<td>3948.0 ± 418.6</td>
<td>3518.0 ± 2.8</td>
</tr>
</tbody>
</table>

Data are mean ± SD.

* Reduced transcription not seen in this group.
† Erb2 proto-oncogene and GNAS were increased less than 1.4-fold in this group, but were increased more than 1.4-fold in the uninfected TGs, and therefore are included to achieve a balanced composition in the ANOVA.
‡ Prothrombin beta 4 and brain lipid-binding protein were increased less than 1.4-fold in this group, but were increased more than 1.4-fold in the uninfected TGs, and therefore are included to achieve a balanced composition in the ANOVA.
FIGURE 1. Each phosphorimage is a representative example of microarray analysis of polyA+ mRNA from the TGs of mice. (A) Uninfected mice, not treated; (B) uninfected mice, drug treated. cDNA array panels are divided into seven theme-targeted sectors, including (a) basic transcription factors, (b) cell-cycle regulation, (c) oncogenes, (d) chemokine-cytokine signaling, (e) signal transduction, (f) cytoskeleton proteins and DNA damage repair proteins, and (g) housekeeping genes. Arrows: cDNA spots that show significant increases in expression.

TABLE 2. Mouse-Specific Primer Sequences and Sizes of RT-PCR Products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Orientation</th>
<th>5'-3' Sequence</th>
<th>Sizes of Amplified Fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>5'-TGAAGGTCCGGTGGTGAAGCGGGTTTGGC-3'</td>
<td>983</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-CATCGTGCCGATCGAGCTCAGACCCGAG-3'</td>
<td></td>
</tr>
<tr>
<td>PTGER4</td>
<td>Sense</td>
<td>5'-TTCCGCGTGGCGGCGAGTTCTTGGTCA-3'</td>
<td>423</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-AGAGGCTGTCAGCGACACGAGAGC-3'</td>
<td>434</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>Sense</td>
<td>5'-TTCCGCGTGGCGGCGAGTTCTTGGTCA-3'</td>
<td>423</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-AGAGGCTGTCAGCGACACGAGAGC-3'</td>
<td>434</td>
</tr>
<tr>
<td>Inhibitor of DNA-binding protein 4</td>
<td>Sense</td>
<td>5'-CTTGTCAGACACCTTGTCACGTCGAC-3'</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-GGTGGTGTCTGCTTGGGTCAGCGAGAGC-3'</td>
<td></td>
</tr>
<tr>
<td>Transcription factor AP-1</td>
<td>Sense</td>
<td>5'-ATGCCCTCAACGCCTCGTTCCTCC-3'</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-CTGCTCGTCGGTCACGTTCTTGGG-3'</td>
<td></td>
</tr>
<tr>
<td>Myelin protein zero</td>
<td>Sense</td>
<td>5'-ACTATGGCGACAGGGACACTTTAGTC-3'</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-ACATAGAGCGTGACCTTGACAGAGTC-3'</td>
<td></td>
</tr>
</tbody>
</table>

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Data Analysis

Signal intensity data were obtained from two independent cDNA array panels with four groups by phosphorimaging onto molecular imaging screens, by using a molecular imager (model GS250; Bio-Rad, Hercules, CA). The labeled cDNA was hybridized to the array membrane for 18 hours, followed by 48 hours of exposure to the storage screens. The data were analyzed using software for Atlas array analysis (AtlasImage 2.0; Clontech). The signal intensities on a control membrane (no drug treatment) and an experimental membrane (drug-treated) were normalized by a global method, according to the software user’s manual. The intensity obtained was compared between two groups (no drug treatment versus drug treated). Signal analytical parameters (signal ratio, signal difference, and common cDNA filter background correction) were adjusted so that gene targets exhibiting the largest nonimmunosuppression-to-immunosuppression changes exceeding a factor of 1.4 or more were reported. In one published study, a postnormalization cutoff of a twofold increase or decrease in measured level was used to define differential expression, although there is no established theoretical basis for selecting this level as significant. For statistical analysis, any values that were increased or decreased 1.4-fold or more in either comparative group were used. Next, analysis of variance (ANOVA) was performed to assess the statistical significance of the genes listed in Table 1. Statistical evaluations of the magnitude and direction of change in gene expression levels were conducted by two-level factorial ANOVA and followed by two-tailed protected t-tests. The factors (main effects) in this ANOVA model were immunosuppressive drug treatment and viral latency (+ or −). The Tukey method was used to correct the significance criterion levels for the number of multiple comparisons made.

Semiquantitative RT-PCR
cDNA was synthesized using reverse transcriptase (SuperScript II; Gibco BRL, Carlsbad, CA), according to the manufacturer’s recommendations. Reverse transcription was performed using 5 µg RNA and 0.5 µg oligo(dT) primer. Table 2 lists sequences of mouse-specific oligonucleotide primer pairs. The cDNA sample was amplified in a buffer containing 1.5 mM MgCl₂ (Perkin-Elmer, Oceanport, NJ), 0.2 mM of each dNTP (New England BioLabs, Beverly, MA), 0.5% DNA polymerase (AmpliTag; Perkin-Elmer), and 0.2 µM of each primer pair. The conditions for PCR were as follows: an initial 10-minute denaturing step at 94°C and exponential cycles of 30 seconds at 94°C, 30 seconds at 52°C, and 1 minute at 72°C. The PCR products were examined by 2% agarose gel electrophoresis with ethidium bromide staining. To determine the relative levels of gene expression, semiquantitative
analysis was performed by the method reported in Nakayama et al. and Yokoi et al. The optical density of each band was measured, and the background intensity was subtracted from the band density by using image-analysis software (Eagle Sight 3; Stratagene, La Jolla, CA). For estimation of the initial amount of the template, the equation $\frac{y}{H_{1005}} = ax + b$, where $y$ is the logarithm of the PCR product’s intensity, $a$ is the efficiency of amplification every cycle, $x$ is the number of cycles, and $b$ is the initial amount of the template, was fitted to the data in the linear portion of the graphs. The ratio of the initial amount of each gene to GAPDH was compared for each sample. These experiments were repeated at least twice for each sample and primer pair.

RESULTS

Microarray

The results of the microarray phosphorimaging of uninfected and latently infected TGs are shown in Figures 1 and 2: uninfected, non-immunosuppression (Fig. 1A); uninfected, immunosuppression (Fig. 1B); HSV-1 latently infected, non-immunosuppression (Fig. 2A); and HSV-1 latently infected, immunosuppression (Fig. 2B). Signals were not seen in the negative control spots, indicating the one area of specificity of the hybridization. To determine the reproducibility of the results, a series of experiments was performed twice (eight hybridizations to eight microarray membranes). The results were remarkably similar, and the signal intensity readings for each gene were averaged from the two experiments. The lists of statistically significant (ANOVA, protected $t$-test, $P < 0.05$) genes with expression that increased or decreased greater than 1.4-fold are presented in Table 1. Two genes in the TGs of uninfected, drug-treated mice exhibited increased expression. The TGs of latently infected, drug-treated mice showed increased expression of 10 genes and reduced expression of 8 genes.

Semi-quantitative RT-PCR

Five genes whose transcriptional level was altered by the drug treatment were analyzed by semi-quantitative RT-PCR. The genes studied were inhibitor of DNA-binding protein 4 (ID4), PTGER4, cyclin D2, transcription factor activator protein (AP)-1, and myelin protein zero. Semi-quantitative RT-PCR yielded results consistent with microarray results for each of the five genes studied (Figs. 3, 4; Table 3).

DISCUSSION

Our ultimate goal is to prevent reactivation of HSV-1 and recurrent disease by inhibiting or stimulating host genes required for ocular recurrence of HSV-1. In this study, the transcriptional activity of 10 genes was significantly increased, and the transcriptional level of 8 genes was decreased in latently infected mice treated with cyclophosphamide and dexamethasone, compared with untreated latently infected mice.

The involvement of cellular proteins in HSV latency and reactivation has been reviewed. Some of the host cell proteins thought to be important in latency and reactivation have been noted in the introduction. By microarray analysis, we showed previously that nine genes including transcription factor II, DNAJ-like heat shock protein, stress-activated c-jun kinase 3 (JNK3), and COX-2 underwent transcriptional activation 1 hour after hyperthermia.
The results of the present study and other published observations indicate that PTGER4, cyclin D2, transcription factor AP-1, mitogen-activated protein kinase (MAPK), and IPF1 could have important roles in HSV-1 infection and reactivation. The expression of these genes is thought to be altered in response to immunosuppressive induction of uninfected or HSV latently infected neurons or viral replication after reactivation. To determine this, we must investigate when viral and cellular genes begin to change after induction of immunosuppression. Furthermore, it is difficult to determine in the current experiments whether induction of immunosuppression was caused primarily by the cyclophosphamide or primarily by the dexamethasone. It is possible that neither cyclophosphamide nor dexamethasone alone could reactivate latent HSV. Stroop and Schaefer reported that injection of dexamethasone or cyclophosphamide alone did not reactivate latent HSV-1 in rabbits; only 3% and 1% of eyes were positive for HSV-1 in rabbits receiving cyclophosphamide or dexamethasone, in contrast to the reactivation frequency of 85% observed when the drugs were administered together. They suggested that these two drugs act in a synergistic manner. If changes in gene expression are examined only after injection of cyclophosphamide or dexamethasone, fewer genes may be altered than were changed in the present study. Naray-Fejes-Toth et al. reported that an initial effect of dexamethasone was to increase the expression of the glucocorticoid-induced.

**Table 3.** Density Ratios of Untreated and Drug-Treated HSV Latently Infected TGs by Semiquantitative RT-PCR and Microarray

<table>
<thead>
<tr>
<th>Gene</th>
<th>Uninfected</th>
<th>Latent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>Microarray</td>
</tr>
<tr>
<td>ID4</td>
<td>0.74 ± 0.13</td>
<td>0.84 ± 0.15</td>
</tr>
<tr>
<td>PTGER4</td>
<td>1.07 ± 0.02</td>
<td>1.25 ± 0.24</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>1.33 ± 0.39</td>
<td>1.46 ± 0.19</td>
</tr>
<tr>
<td>Transcription factor AP-1</td>
<td>0.64 ± 0.11</td>
<td>0.66 ± 0.46</td>
</tr>
<tr>
<td>Myelin protein zero</td>
<td>0.61 ± 0.10</td>
<td>0.53 ± 0.18</td>
</tr>
</tbody>
</table>

Data are mean ± SD.
kinase (SGK) gene. Time course analyses revealed that SGK mRNA levels are elevated as early as 30 minutes after exposure to the glucocorticoid, and the levels remain elevated for several hours. Using this observation, we selected the drug treatment protocol used in this study.

We noted a significant increase in expression of the PTGER4 gene, which codes for a prostaglandin receptor. In this study, expression of the PTGER4 gene was increased 25-fold in latently infected TGs after drug treatment and only 1.2-fold in drug-treated uninfected TGs. Although microarray analysis showed that expression of PTGER4 was increased 25-fold in drug-treated, latently infected TGs, semiquantitative RT-PCR indicated only a 3.05-fold upregulation of gene expression. According to previous reports, the changes in gene expression obtained from an array system are usually similar to, but are not always identical with, those obtained from semiquantitative RT-PCR. Glucocorticoids attenuate expression of COX-2, and deregulated expression of COX-2 leads to an overproduction of prostaglandins. Also, perhaps the upregulation of this gene is a host response to reactivation of HSV-1, similar to that seen in acute cytemegalovirus infection.

The expression of the cyclin D2 gene was increased to similar levels after drug treatment of uninfected and latently infected mice. Cyclin D consists of three subtypes of cyclins (D1, D2, and D3). Cyclin D2 is implicated in cell cycle regulation, differentiation, and oncogenic transformation. Cellular cyclin-dependent kinases (CDKs) are required for HSV replication. Inhibitors of CDK-1, -2, and -3 inhibit viral replication. Because members of the cyclin D family bind to and activate CDK-4 and CDK-6, inhibitors of these genes may prevent HSV-1 reactivation.

In this study, the genes for transcription factor AP-1 and MAPK-3 exhibited reduced transcription after immunosuppression. AP-1 can regulate many aspects of cell physiology in response to environmental changes. Fos and Jun constitute the prototypic components of the AP-1 complex. MAPKs have been implicated in many physiological processes and phospholipid-Jun proteins. Acute HSV-1 infection, bacterial infection, and emotional stress activate p38/c-Jun N-terminal kinase, cyclin F, transcription factor S-II, and glutathione S-transferase. In contrast, myelin gene expression decreased. Although the investigators studied uninfected TGs and ex vivo events, some of the same genes, such as PTGER4, glutathione S-transferase μ2, cyclin D2, PMP22, and transcription factor AP-1, were altered in their study as in our present study of HSV-1-latent TGs induced in vivo.

HSV-1 can be induced to reactivate by UV irradiation, trauma, heat stress, cold stress, and adrenergic iontophoresis. To better understand the interactions between the host and the virus, we plan in future experiments to investigate changes in gene expression after the use of these other inducers.

In summary, 18 genes were significantly altered after injection of cyclopophamid and dexamethasone in mice latently infected with HSV-1. One or more of these genes could hold the key to the prevention of recurrence of ocular herpes. If substances that regulate these genes can be shown to block viral reactivation, such agents could form a new class of antiviral medication.

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References


