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ORIGINAL ARTICLE

Gene Expression Profiles in GP160-Expressing Mouse Model of HIV-Associated Dementia

Masoud Ghorbani[1,2*], Sedigheh Hatami Gigloo[1]
[1] Pasteur Institute of Iran, Research and Production complex, Kilometre 25 Autobane karaj, Iran.
[2] Infectious Disease and Vaccine Research Center, Children's Hospital of Eastern Ontario Research Institute, 401 Smyth Road, Ottawa, ON, K1H 8L1, Canada.
Email: mghorbani@irimc.org
Phone: +98-9102104228

ABSTRACT
To investigate the gene expression profile changes in transgenic mice expressing the gp 160 HIV envelope proteins in neurons using cDNA microarray. The microarray data were generated by 15 K mouse cDNA arrays from the Toronto Microarray Centre (Toronto, Ontario) and analyzed by bionormalizer and biomining tools developed at the national research council of Canada. A comparison between control mice and gp 160- expressing transgenic mice identified 57 upregulated and 70 dowregulated genes, which showed at least 1.5 fold change in expression with p<=0.001. The major alterations in gp 160 expressing transgenic mice included changes to long- term potentiation signalling pathways, apoptotic pathways, calciumsignalling, glutamate homeostasis and signaling, protein metabolism, and neurotransmission. Conclusions: the results of the present study provide important leads for pursuing a more complete understanding of the molecular events of gp 160 expressions in neurons.
Key Words: *HIV, gp160, Microarray, HIV-associated dementia*
Abbreviations: HIV=HIV-1; HAD= HIV-associated dementia; GO= Gene Oncology; LTP=Long-term Potentiation

INTRODUCTION

HIV type I (HIV-1) infection causes neurodegeneration in patients with AIDS [1]. The morphological manifestations of this neurodegeneration are collectively designated HIV-1-associated dementia (HAD) [2]. Autopsy examination of patients who died of HIV disease revealed neuropathological changes in as many as 80% of infected patients [3]. In fact, HIV infection is the leading cause of dementia in the 20-60 age groups [4], with more than 20% of HIV patients developing HAD [5]. Furthermore, the advent of antiretroviral therapy has improved survival rates, increasing the prevalence of a milder form of HAD in which cognitive dysfunction is more pronounced.

Cognitive deficits, manifested by mental slowing, forgetfulness, and difficulty in concentration, emerge early in the course of disease and are the most frequent symptom complex seen in HAD [6]. Early motor symptoms include unsteady gait, limb weakness, and tremor. Frequently, a host of behavioural symptoms ensue [7], culminating in dementia and paralysis. At the cellular level, the pathological hallmark of HIV-1 infection of the brain is the presence of multinucleated giantcells which are formed by syncytia of HIV-1 infected macrophages [8]. Neurons undergo dendritic pruning [9], loss of synapses [10], and cell death [11]. Morphological manifestations include HIV-1 encephalitis (HIVE) and HIV-1 leukoencephalitis (HIVL) [12].

There are two postulated mechanisms of neuronal damage by HIV-1 infection: direct and indirect. Since neurons themselves are either not infected by HIV or are infected at undetectable levels, it is postulated that the neuronal effects of HIV-1 are mediated indirectly through perivascular macrophage and microglia, the two predominant cell types infected by HIV in the CNS. Infected cells release neurotoxins (e.g. glutamate, pro-inflammatory cytokines, chemokines, nitric oxide, and other cellular factors) which activate the N-methyl-D-aspartate (NMDA) receptor- mediated intrinsic apoptotic pathway.

Evidence also supports direct damage by HIV-1 proteins, including HIV-1 envelope glycoprotein gp 120, tat, and Var, in the induction of neuronal injury. The effect of gp 120 has been associated with an intracellular increase in free calcium, likely mediated by excessive activation NMDA receptor-operated channels. Thus, neuronal damage and apoptosis is likely incurred by parallel mechanisms

whereby HIV-1 proteins either induce neurotoxin release by infected macrophages and microglia and/or through direct contact with neurons [12].

In the present study, we compare the gene expression of NFL-gp160 mice and wild-type mice by microarray analysis. The gp 160 transgenic mouse expresses the HIV *env*gene in neurons under the control of the human neurofilament (NFL) promoter. The *env* gene encodes the gp 160 protein, which is cleaved to form the neurotoxic envelope glycoproteins gp 120 and gp 41. This model was developed to examine the direct effects of gp 160 protein in neurons, which in the past has been difficult to elucidate due to the systemic effects of the HIV-1 infection process [13]. The gp 160 transgene is predominantly expressed in anterior thalamic and spinal motor neurons. Animals developed a neurological syndrome characterized by hypoactivity and weakness and by axonal degeneration in peripheral nerves. This model offers a unique opportunity to investigate the role of the gp 160 protein in HAD.

MATERIALS AND METHOD

Animals

NFHgp160 mice are descendants of the founder murine line NFH1932. They express the gp 160 transgene in neurons under the control of a murine neurofilament light (NFL) promoter as described in Michaud et al 2001. The control mice in these experiments are B6C 3F1 purchased from Charles River (St. Constant, Quebec, Canada). Mice were sacrificed by CO2 inhalation, and their brainstems and cerebella were rapidly dissected and stored at -80 c. the mice were housed and cared for in a fashion approved by the Animal Care Committee of The Hospital for Sick Children.

RNA Preparation

Total RNA was extracted from a homogenated of brain stem and cerebellum using RNeasy Lipid Mini Kit (Quiagen, Mississauga, Ontario Canada) as per the instructions of the manufacturer.

Labeling and Hybridization

Five biological samples from different NFLgp 160 and control mice were run in triplicate were used for microarray analysis. Between 10 and 15 µg of RNA was reverse-transcribed using SuperScript II RNase H Reverse Transcriptase (Life Technologies), 100 pmol/µL Anc-T primer (Invitrogen Ontario, Canada), and a random hexanucleotide (Cortec DNA Service Laboratories) in the presence of 6.67 Mm dNTP-dCTP and 2 µmol/L dCTP (Amersham Pharmacia Biotech, Baie d, Urfe, Quebec, Canada), and 1 µL either Cy3-dCTP or Cy5-dCTP (Perkin Elmer). The reaction was allowed to proceed for 2 hours at 42 C. to stop the reaction, 4 µL of 50 mM EDTA and 2 µL of 10 N NaOH were added to hydrolyze the remaining RNA and added 4 µL of 5 M acetic acid to neutralize the mixture. The Cy3 (wt) and Cy5 (gp 160) reaction mixtures were combined and the labelled cDNA collected with the use of Millipore Microcon polymerase chain reaction filter units (Fisher Scientific, Nepean, Ontario, Canada). One experimental replicate per biological sample was reciprocally labelled.

The hybridization solution was a 20:1:1 ratio of DIG Easy Hyb (Roche, Mississauga, Ontario, Canada), sonicated calf thymus DNA (Sigma-Aldrich, Oakville, Ontario, Canada), and yeast tRNA (Life Technologies). 5µL of the combined Cy3/Cy5 sample was added to 80 µL of hybridization solution and was incubated at 65C for 2 minutes and was then left to cool to room temperature. The sample wasloaded onto the double-spotted, two channel mouse cDNA microarray (Toronto Microarray Centre, Ontario, Canada) and incubated in a hybridization chamber overnight at 37 C. after hybridization ,the coverslip was removed in 1X SCC. The microarray was washed three times for 12 minutes each at 50C in clean slide staining boxes containing pre-warmed 1X SCC, 0.1% sodium dodecyl sulphate (SDS) with gentle agitation.

The slide was successively rinsed at room-temperature with 1X SCC, 0.1 X SCC, and distilled water and dried by centrifugation at 500 rpm for 5 minutes and stored in the dark.

Quantitation, Background Subtraction, Normalization and Data Filtering

Each microarray was scanned using a Scan Array XL 4000 fluorescent scanner (Packard Bio-chip, CA), yielding two 16-bit images corresponding to the Cy3 channel and Cy5 channels. Image intensity was quantitated and background was subtracted using Scan Array Express (Packard Bio-chip, CA),a pre-processing tool.

Data was obtained from 15 cDNA microarrays, consisting of five biological replicates and three technical replicates. In total, each microarray consisted of 15 600 cDNA probes, each spotted in

duplicate. Background was subtracted from spot intensities using the following formula: Corrected Spot Intensity = Spot Intensity – Signal to Background Ratio x Mean Background Intensity. Low intensity, where the background-corrected intensity value is less than a preset Low Intensity Threshold (LIT), were replaced by a preset LIT of 5.

Low quality spots were flagged on each microarray based on (1) signal-to-background ratio <2.5; (2) intensity < 5 percentile in either channel; (3) intensity > 98 percentile in either channel; (4) visual identification of low quality spots during quantitation. Intensities were converted to a logarithmic ratio (log2 [transgenic/ wild type]).

By dominancy analysis, the intensities of identical probes across each biological replicate were consolidated. Thus, six probes (three technical replicates with probes spotted in duplicate) representing one cDNA were examined for each biological replicate. Where at least four out of six intensities were similar, an average of the intensities of the dominant subgroup was averaged and used to represent the intensity of that probe. Where this threshold was not met, i.e. disparate intensities were observed for more than two out of six identical probes; the gene was discarded and excluded from subsequent analysis.

Data from each biological replicate was normalized using BioNormalizer, a tool developed at the National Research Council of Canada (NRC). Low quality samples were excluded from normalization.

RESULTS

In total, 15 microarrays, consisting of three technical replicates and five biological replicates were analyzed. Data from each microarray underwent background subtraction, quantitation, normalization, and filtering, resulting in a total of 13 002 high quality gene for final analysis (see Table 1).

Bad spot filtering, resulted in a total of 13 002 genes for analysis. T-test was used to identify differentially expressed genes. P<=0.001 and fold change >=1.5 were taken to be statistically significant. T-test revealed 129 differentially expressed genes, with 57 gene being upregulated and 70 gene being downregulated.

These differentially expressed genes were further underwent GO clustering by biological process, molecular function, cellular component, and by all GO terms. Of the 129 differentially expressed genes, 90 have been characterized by gene ontology (see Table 1). Gene ontology clustering showed statistically significant differential expression of genes involved in long-term potentiation pathways (LTP), suggesting that direct contact between the gp 160 HIV envelope protein and neurons can depress long-term potentiation, thereby causing the cognitive defects observed in HAD. Additionally, we observe differential expression of transcription factors, signal transduction systems, calcium ion homeostasis dysfunction, and neurotransmission deficits.

DISCUSSION

Experimentation has definitively shown HIV infection of glia and macrophages in the CNS. However, evidence of neuronal infection by the HIV virus has been limited, possibly due to low and undetectable expression of viral gene products following neuronal HIV infection. To further investigate this theory, a mouse model was created which expresses the gp 160 transgene in neurons. These mice exhibit neurological pathologies very similar to those found in patients with HIV-associated dementia (HAD), including alterations of the neuronal cytoskeleton, vacuolization of dendritic processes, axonal swelling, and degeneration of synapses, dendrites, and axons [13]. The morphological changes affect cognition in patients with HIV-1 by causing forgetfulness, apathy mental slowing, and difficulty concentrating, which progress to dementia.

Microarray analysis of global gene expression was undertaken to determine cellular and molecular mechanisms that may contribute to HAD. In total, 129 genes were differentially expressed with a p value <=0.001, of which 57 were upregulated and 72 were downregulated. This implies that a large number of the surveyed genes were affected by gp 160 exposure. The up-regulation or down-regulation of these genes may be responsible for gp 160-induced neural pathologies.

Aberrant Expression of Genes involved in Glutamate Metabolism and Signalling

The Glud 1 gene which encodes the glutamate metabolizing enzyme glutamate dehydrogenase I was shown to be downregulated in gp 160-expressing mice. Abberent glutamate signaling has been

linked to many neurodegenerative diseases, including HAD. Since the levels of glutamate are largely determined by the rate of its metabolism, the decrease d expression of glutamate dehydrogenase I, which converts glutamate to alpha-ketoglutamate, would result in increased levels of intracellular glutamate. This has been associated with temporal lobe epilepsy [14], while elevated glutamate dehydrogenase activity has been associated with Alzheimer [15] disease and schizophrenia [16]. Clearly, glutamate metabolism and the glutamate-glutamine cycle are of fundamental importance in neurodegenerative disease. The significance of the downregulation of GDH in the present study still needs to be investigated further.

Grid2 encodes the ionotropic delta2 glutamate receptor. These receptors play a central role in synaptic plasticity, neuronal cell death, and certain neurodegenerative disease. This receptor, which is predominantly expressed in Purkinje cells, is crucial for cerebellar function. Null mice display ataxia and impaired learning. Its upregulation in gp 160 transgenic mice is paradoxical, and may be a compensatory response to neuronal damage caused by HIV infection.

Aberrant Expression of Genes Involved in Signal Transduction

Our results support the theory that alteration to the neuronal circuits involved in long term potential may be the underlying mechanism causing the dementia present in HIV-1-infected patients. Rho family of small GTPases is regulator of both the action cytoskeleton and the LTP process through their regulation of the morphological development of neurons, including axonal growth and guidance, dendritic elaboration, and formation of synopses. In neuronal systems, RhoGAPs, encoded by ARHGAP family genes, are negative regulators of Rho family GTPases. The upregulation of Arhgap9 and Arhgap12, whose protein products hydrolyse Rho proteins therefore deters from the process of LTP, and thus may play a role in the cognitive impairment present in HAD.

Our finding that LTP can be hindered by exposure to the gp 160 HIV envelope protein is supported by other studies. The gp 120 subunit of gp160, in conjunction with GABAA receptor antagonist, has been shown to inhibit LTP in a concentration-dependent manner [17]. As well, gp 120 exposure in rats caused poorer performance in the Barnes maze task and impaired the induction and maintenance of LTP.

Anp32e is upregulated in gp 160-expressing transgenic mice. The protein product of this gene inhibits protein phosphatase 2A (PP2A). PP2A is a modulator of CaMKII activity, an essential enzyme for LTP and spatial memory. In knockout mice lacking PP2A, mice show impaired memory and learning. In fact, it has been shown that modulation of even a small percentage of CaMKII activity by PP2A in vivo in enough to impairment of learning and memory [18].

Calcium Signalling

The results also show that the calm2 gene is downregulated in our HAD model. The calm2 gene encodes the protein calmodulin, a calcium-banding protein. Calmodulin plays a vital role in LTP by modulating the activity of calcium and by stimulating many of the enzymes involved in memory formation, including calcium/calmodulin-dependent protein kinase II (CaMKII), adenylyl cyclase 1 and 8, and calcineurin [19]. Lack of stimulation by calmodulin has been shown in previous studies to impair memory and learning. Furthermore, calmodulin/calcium stimulates nitric oxide sythase (NOS), which produces nitric oxide (NO), a retrograde reinforce of LTP. In the absence of NO, memory formation is severely impaired.

Decreased Expression of Genes Involved in Synaptic Function

A pronounced alteration identified in transgenic gp160 mice was the differential expression of genes involved in the depression of synaptic neurotransmission. Among them, SNAP-29 has been previously shown to act as a negative modulator for neurotransmitter release, purportedly by slowing recycling of the SNARE-based fusion machinery and synaptic vesicle turnover [20]. Its upregulation suggests a deficit in neurotransmission.

Synaptotgamin2 was observed to be upregulated in transgenic gp 160 mice. Synaptotagmin2, a presynaptic calcium sensor, mediates synchronous fusion of synapses. The significance of its upregulation in transgenic gp 160 mice is potentially indicative of signalling aberrancy. The consequences of this remain to be fully elucidated.

Apoptosis Genes

Genes involved in the cytochrome c apoptosis pathway were found to be downregulated including cytochrome c and cytochrome c oxidase. Additionally, cox7c which encodes a subunit of

cytochrome c oxidase, and uqcrh, a mitochondrial hinge protein which accelerates apoptosis by enhancing the release of cytochrome c from mitochondria, were also downregulated.

Interestingly, Stage 1, a key mediator of p53 dependent apoptotic pathway [21] was up regulated. Taken together, this suggests that apoptosis induced by gp 160 exposure occurs via a p53 pathway and not via a cytochrome c oxidase pathway.

Alteration in Genes Involved in Protein Metabolism

Among the genes that play a role in protein metabolism, ADAM 8 and ADAMTS 19 were significantly downregulated. ADAM and ADAMTS protein are a family of metalloproteases involved in the cleavage of transmembrane proteins. Some members of the ADAMTS family have been implicated in disruption of the preineuronal matrix [22]. However, the question of whether other membranes of the metalloproteases such as ADAM8 and ADAMPTS 19 participate in ECM destruction and the biological relevance in the downregulation in HAD remain to be investigated.

In summary, we observed differential expression of transcription factors and the signal transduction system, which may result in neuron apoptosis, LTP dysfunction, and calcium ion homeostasis dysfunction, and neurotransmission deficits. A number of the candidate genes we describe have not been previously linked to HAD, and thus define novel injury pathways warranting further study. The differentially expressed genes uncovered in the present study provide clues for pursuing a more complete understanding of the molecular mechanism of gp 160-induced neural degeneration and memory impairment.

Table 1 – Differentially Regulated Genes and Their Major Function

Major Function	Gene Symbol	Fold change	P value
Actin/ cytoskeleton- related	Adam 8	1.660	0.00055
	Catna 3	**1.907**	**0.00068**
	Catnal 1	**2.598**	**0.00160**
	Cnn 1	**1.529**	**0.00362**
	Adamts 19	**1.588**	**0.00420**
	Coro2b	**1.836**	**0.00564**
	Robo 1	**3.584**	**0.00666**
	Krt1-18	**2.391**	**0.00842**
Cell death	**Cycs**	**1.683**	**0.00011**
	Stag 1	**2.360**	**0.00071**
	Mmd 2	**2.312**	**0.00329**
	Uqcrh	**2.070**	**0.00384**
	Cox7c	**2.187**	**0.00843**
Cell motility	**Actr2**	**2.495**	**0.00292**
Cell projection biogenesis	**Capg**	**2.094**	**0.00505**
Chromatin assembly/disassembly	**Myst 1**	**1.538**	**0.00161**
Electron transport	**Acads**	**2.612**	**0.00253**
Embryonic	**Pace 4**	**1.817**	**0.00844**
Gluconeogenesis	**pck**	**1.652**	**0.00731**
Growth	**Gpc 3**	**1.691**	**0.00137**
Immune response	**Gmfb**	**1.663**	**0.00438**
Ion binding	**Daf 1**	**1.849**	**0.00890**
	Spna 2	**1.653**	**0.00586**
	Slc14a1	**1.600**	**0.00787**
	Slc25a30	**2.406**	**0.00916**
Ion transport	**Kcns3**	**2.133**	**0.00420**
	Slc20a1	**1.869**	**0.00786**
	Atp5b	**1.540**	**0.00694**
Lipid metabolism	**Stard 5**	**3.033**	**0.00099**
	Abca 3	**2.014**	**0.00191**
	Liph	**1.941**	**0.00470**
	Scp2	**1.807**	**0.00741**
Motor impairment	**punc**	**1.850**	**0.00003**
mRNA processing / splicing	**Rbm14**	**1.601**	**0.00094**
	Hnrpa0	**1.583**	**0.00245**
	2600011c06Rik	**1.537**	**0.00527**

Neurotransmission	D12Ertd551e	1.753	0.00148
	Anp32e	1.643	0.00459
	Vps11	1.976	0.00505
	Snap29	1.976	0.00505
	Azin1	1.739	0.00354
	Rpp30	1.894	0.00417
	Trib1	1.758	0.00516
	Srpk1	2.077	0.00978
Protein binding	Hspa5	1.917	0.00325
Protein metabolism	Usp48	2.598	0.00022
	Rnf14	2.140	0.00059
	201010G01Rik	1.642	0.00073
	Rp17	1.701	0.00098
	Rnf128	2.188	0.00147
	Rp16	1.653	0.00183
	Rrbp1	2.613	0.00244
	Rpl15	1.810	0.00372
	Senp1	1.837	0.00419
	Ubr1	1.729	0.00536
	Espl1	1.741	0.00651
	Mbtps1	1.795	0.00815
	Arih1	2.163	0.00863
Protein targeting	Srp54	2.040	0.00025
	Ywhaz	1.738	0.00082
	Arl6ip1	1.668	0.00509
Protein transport	Vps28	1.639	0.00068
	Slc15a2	1.673	0.00527
	Sec23b	2.368	0.00762
	Ap3s2	2.100	0.00919
Regulation of transcription	Zfp57	2.079	0.00051
	Ddx5	2.411	0.00156
	Hipk3	2.105	0.00461
	Zfp52	2.028	0.00529
	Hdac2	2.853	0.00594
	E2f7	2.082	0.00646
	Zfp198	1.557	0.00649
	Psx1	1.743	0.00705
	Chd8	1.625	0.00728
	Tgif2	1.930	0.00756
	Twist2	1.759	0.00823
	Hira	2.200	0.00874
	Chd4	2.695	0.00932
Signal transduction	1300019c06Rik	1.796	0.00027
	Phip	2.577	0.00044
	Arhgap12	2.371	0.00054
	Tbc1d15	2.113	0.00186
	Grid2	1.899	0.00269
	Ptprs	1.778	0.00315
	Pdgfrb	1.779	0.00389
	Arhgap9	1.805	0.00454
	Glud1	1.810	0.00580
	Gkap1	1.701	0.00834

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