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Elucidating effects of long-term expression of HIV-1 Nef on astrocytes by microarray, promoter, and literature analyses

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Abstract

The challenge of microarray analysis is to unveil the biological mechanisms behind the chip data. Due to the sometimes counteracting influences of de novo transcription, RNA processing and degradation, the discovery of any particular mechanism is difficult. Therefore, a combination of data- and knowledge-driven analysis appears to be the best way to attack the problem. We analyzed human astrocytes stably expressing the HIV-1 *nef* gene by microarray analyses to elucidate the effects of constitutive HIV-1 Nef expression on the transcriptome of astrocytes. Statistical evaluation of microarray results revealed small clusters of genes specifically up-regulated by native Nef protein in contrast to astrocytes expressing a non-myristoylated Nef variant. At least three significantly overrepresented gene ontology groups (small GTPase signaling, regulation of apoptosis and lipid metabolism) were detected. The JAK/STAT pathway was clearly associated with those genes. This finding agreed well with a literature-based approach, where a network was derived by combined literature and promoter sequence analysis. Promoter organization suggested potentially coordinated transcriptional regulation of some of these genes. Both results were in line with previously reported phenotypic changes.

Keywords: Astrocyte activation; Gene ontology; Pathway analysis; JAK/STAT

1. Introduction

The immune system and the central nervous system contain various target cells for HIV-1. These include CD4-positive T-cells and macrophages as well as microglia cells and astrocytes (for reviews see Stebbing et al., 2004; Gonzalez-Scarano and Martin-Garcia, 2005). The HIV-1 Nef protein is an early viral protein expressed together with other HIV-1 regulatory factors before production of HIV-1 progeny particles. Nef has pleiotropic activities which increase the pathogenicity of the virus in the host by modulating cellular properties (reviewed in Greenway et al., 2003). One of the functions reported for Nef is the activation of CD4-positive T-cells, which has been proposed to promote dynamic virus production by these cells (Simmons et al., 2001).

Expression of Nef can also occur during persistent infection of cell types that limit HIV-1 replication and form HIV-1 reservoirs (reviewed in (Blankson et al., 2002)). One example for a Nef-expressing cellular HIV-1 reservoir is the astroglial population, which is the most abundant cell type in the CNS. Astrocytes form a pool of persistently infected cells in the brain. Expression of Nef has been observed in astrocytes both in postmortem brain tissues from individuals with AIDS (i.e. in vivo) as well as in astrocytic HIV-infected cell cultures (reviewed in Brack-Werner, 1999; Gorry et al., 2003). The HIV-1 Nef protein is post-translationally

Abbreviations: CNS, central nervous system; CD, cluster of differentiation; GO, gene ontology; G418, Geneticin; GTPase, guanidin triphosphatase; NCBI, National Center for Biotechnology Information; HIV-1, Human Immunodeficiency Virus-1; *rev*, *nef*, *tat*, HIV-1 encoded regulatory and accessory genes; Rev, Nef, Tat, corresponding HIV-1 encoded regulatory and accessory proteins; TFBS, transcription factor binding site.

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modified by addition of myristoylic acid to the N-terminus and this myristoylation was found to be essential for several Nef functions (Peng and Robert-Guroff, 2001).

To investigate the effects of long-term Nef expression on the properties of human astrocytes we established several stably Nef-expressing sublines of the U251MG astrocytoma cell line (Kohleisen et al., 1999). We have previously reported that sublines expressing wild-type Nef show morphological alterations, increased expression of activation markers (Kohleisen et al., 1999) and increased tumorigenicity in nude mice (Kramer-Hammerle et al., 2001). In addition Nef expression has been shown to protect U251MG cells from TNF-alpha induced apoptosis (Robichaud and Poulin, 2000). These observations suggest that stable expression of Nef alters cellular properties of astrocytes.

In this study we analyse Nef-induced changes of gene expression patterns of U251MG cells by combined microarray and pathway analyses. We provide evidence that Nef is capable of inducing similar functional pathways in astrocytes and in immune cells, although the individual molecules triggered by Nef within these pathways may differ. Our results establish a molecular basis for several of the experimentally identified Nef-induced changes in these cells and suggest that persistent Nef expression during HIV-1 infection of astrocytes may lead to constitutive, long-term activation of infected astrocytes.

2. Materials and methods

2.1. Cell culture

The cell line U251MG was established from a human glioma (Bigner et al., 1981). For microarray analysis, U251MG cells stably expressing either Nef derived from the HIV-1 Bru-isolate or the myristoylation-deficient TH-Nef (Kohleisen et al., 1992, 1999) were used. The cells were cultivated in DMEM supplemented with 10% fetal calf serum and antibiotics (1%). For selection of Nef-expressing cells G418 was added at concentration of 500 μg/ml. Nef-expression was confirmed by Western blot analysis of lysates of cells of the same passage number as used for microarray studies (data not shown).

RNA extraction, cDNA synthesis with incorporation of radioactively labeled nucleotides and subsequent hybridization of BD Atlas Plastic Human 12K microarrays was carried out by BD Bioscience Clontech (Palo Alto, CA, USA). This array contains long oligonucleotides representing 11,835 different human genes, including nine housekeeping genes as well as negative and positive controls spotted onto a plastic support (http://www.bdbiosciences.com/clontech/atlas/genelists/index.shtml). The expression profiles of a U251MG sub-line cell clone showing high level Nef expression (U251MG 4/4.2), a pool of cells characterized by moderate Nef expression levels and a cell clone of TH-*nef* expressing U251MG were evaluated. Subsequently each profile was compared to the expression profile of U251MG cell containing only the expression vector with the selection marker for G418 resistance (U251MG pneo, control).

2.2. Array analysis

The BD Bioscience Clontech service included ratio calculation of expression levels in Nef-expressing cells versus control cells, data normalization and a confidence call based on total intensity as well as annotation of the target genes by name, gene symbol and NCBI RefSequence identifier. All further comparisons were based on these ratios, not original expression values.

2.3. Pathway analysis

The BiblioSphere Pathway Edition (Genomatix Software, Munich) collects pathway relevant annotations for single genes from a variety of sources including GeneOntology databases, individual annotation and text books.

2.4. Promoter sequence analysis

We extracted the human promoter sequences from the ElDorado™ database (Genomatix Suite-ElDorado™, release 3.0, Human Genome NCBI build 34, Mouse Genome MGSCv3, Rat Genome NCBI build 2, Genomatix, Munich). The GEMS Launcher task “FrameWorker” using the available weight matrix library (GEMS launcher Version 3.0, matrix library vertebrate section, Matrix Family Library 4.0 containing 535 matrices in 253 families, Genomatix, Munich; http://www.genomatix.de) was used to generate the model of the promoter framework. The FastM task of GEMS Launcher (Klingenhoff et al., 1999) was used to optimize models. ModelInspector (Frech et al., 1997) (a GEMS launcher task) was used to search the human promoter database (Genomatix Promoter Database, GPD, Genomatix, Munich, release 3.0, known genes >36,000 promoters) with the optimized model.

Additional information about connections between the genes from the initial list and candidate genes found by the model search was taken from BiblioSphere™ analyses (Genomatix, Munich, basis for Fig. 4A).

Default parameters were used for the initial analyses in all programs, if not indicated otherwise.

3. Results

3.1. Aim of study

Large-scale expression array analysis of an astrocytoma cell line engineered to stably express Nef was carried out to identify potential Nef-induced molecular changes in astrocytes.