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Original article

Protein expression changes in cells inoculated with Equine Influenza Virus: antibody microarray analysis

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Abstract

Changes in the level of cellular proteins in cells inoculated with equine influenza virus H7N7 and H3N8 were studied with microarray technique. H3N8 induced pro-apoptotic proteins while H7N7 induced both pro- as well as anti-apoptotic factors. The higher level of some cytoskeleton components and proteins involved in the protein quality control was recorded. Relatively high number of proteins involved in the regulation of transcription was down-regulated. The pattern of changes observed for H7N7 and H3N8 may reflect differences in the biological properties of both serotypes.

Key words: equine influenza, influenza, horses, antibody microarrays, proteomics

Introduction

Influenza is regarded as the most important respiratory disease in horses (Powell 1991). Based on antigenicity of hemagglutinin and neuraminidase two serotypes of the equine influenza virus can be distinguished: H7N7 (EIV A1) and H3N8 (EIV A2) (Waddell et al. 1963). There is no evidence of circulation of EIV A1 in horse population after 1980, however EIV A2 serotype is of increasing significance around the world (Bryant et al. 2009, Rozek et al. 2009). Recently, EIV A2 was isolated from dogs and pigs meaning that the virus has crossed the species barrier (Crawford et al. 2005, Tu et al. 2009). Both serotypes have different patterns of multiplication in embryonated chicken eggs and cell cultures (Rozek et al. 1998). These differences could arise from distinct cleavability of hemagglutinin and from specific interactions of viral proteins with cellular components. Identification of differences in the expression level of cellular proteins or their forms after cell infection may contribute to the explanation of some aspects of influenza virus pathogenesis. The analysis of changes in the level of cellular proteins after infection with EIV A1 and EIV A2 was the purpose of the study.

Materials and Methods

We used two strains of equine influenza virus: A/Equi/1/Prague 56 (EIV A1), A/Equi/2/Kentucky 81 (EIV A2) (obtained from the National Veterinary Services Laboratories, Ames, Iowa, USA) and mouse fibroblast cell line 3T3 (from the European Collection of Animal Cell Cultures – ECACC). The virus was propagated in embryonated chicken eggs. The 3T3

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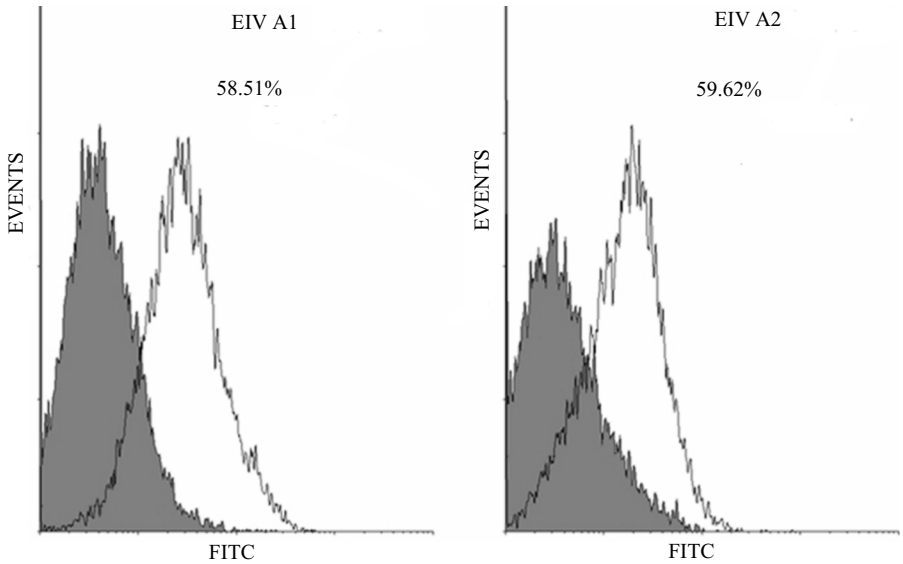

Fig. 1. Flow cytometry analysis of 3T3 cells expressing equine influenza virus antigens. Grey – uninfected control cells, clear – cells infected with EIV A1 (left) or EIV A2 (right).

cells were propagated in Eagle's minimal essential medium with Earle's salts, 2 mM L-glutamine, 1% non essential amino acids and 10% fetal bovine serum. 3T3 cells grown in 75 cc bottles were inoculated with EIV A1 or A2 at $5x10^4$ EID_{50}. Twenty four hours post inoculation the cells were collected and centrifuged at 1,000 g for 15 min and washed in PBS (centrifugation at 500 g, 3 min.). Cells were fixed with Cytofix/Cytoperm kit (BD Pharmingen) according to the producer's instruction. For labeling of infected cells, sera from immunized rabbits (HI 8,192 specific for each of the serotype) and anti rabbit IgG -FITC (Jackson Immunoresearch) were used. Flow cytometry analysis was performed with Epics XL cytometer (Beckman Coulter).

For microarray analysis, one day old 3T3 cell cultures inoculated in the same way as described above for flow cytometry assay were used. Twenty four hours post inoculation the cells were scraped into medium, centrifuged 1,000 g at 4°C for 10 min and washed with PBS. The Panorama Antibody Microarray XPRESS Profiler 725 Kit (Sigma Aldrich) was used to determine levels of cellular proteins in cells inoculated with influenza virus. Microarrays contained 725 antibodies, each printed in duplicate on nitrocellulose-coated glass slides. Protein extraction, labeling and microarray slide processing was performed according to manufacturer's procedures. In brief, cells were lysed in buffer supplemented with protease and phosphatase inhibitors. Proteins were labeled with cyanine dyes (control with cyanine Cy3, infected with cyanine Cy5) and samples were mixed at equal amount (70 µg) before applying to the array. Slides were incubated in the dark for 30 min at room temperature. Then the slides were washed and scanned with ScanArray reader (Perkin Elmer) at 10 µm resolution using wavelengths 550/570 nm for Cy3 and 659/670 nm for Cy5. Results were analyzed with ScanArray Express software (Perkin Elmer). The Lowess normalization method for scanning and the summed fluorescence intensities signal normalization between dyes were used. Normalized mean spot intensities after background subtraction were applied to calculate the relative abundance ratio (fold change) for infected and uninfected samples.

Results

Flow cytometry assay revealed high susceptibility of 3T3 cells to infection with both EIV serotypes. 24 h post inoculation at the dose of 5×10^4 EID_{50}, 58.51% and 59.62% of cells were infected with EIV A1 and EIV A2, respectively (Fig. 1).

Based on microarray analysis, changes in the level of cellular proteins after inoculation were identified. The values above 1.8 and below 0.8 fold change were considered as up- and down-regulation, respectively. The elevated level of 54 proteins (or their forms) was observed in EIV inoculated 3T3 cells: 13 after EIV A1, 35 after EIV A2 and 6 after both EIV A1 and EIV A2 inoculation. Proteins were classified based on their potential role in different biological processes: apoptosis, cell cycle regulation, transcription regulation, protein quality control and cytoskeleton system regulation (Table 1). The level of 34 proteins of 3T3 cells was decreased after EIV inoculation: 18 after EIV A1, 2 after EIV A2 and 14 after both EIV A1 and EIV A2 infection (Table 2).

Table 1. Proteins (gene names) up-regulated in 3T3 cells – comparison of results obtained in cells inoculated with EIV A1 or EIV A2.

Biological process		Proteins up – regulated in 3T3 cells after EIV infection
Apoptosis	EIV A1	Pawr (1.98; P5367)*, Akt1 (2.00; P1601), Diablo (2.11; S0941), Birc5 (2.22; S8191)
	EIV A2	Bcl2l11 (2.01; B7929), Bmf (2.11; B1559), Dapk2 (1.96; D3191), Rybp (1.88; D3316), Tnfrsf10b (2.04; D3938), Fem1b (1.97; F3428), Cflar (2.01; F0305), Perp (2.04; P5243), Prkcb1 (1.87; P4334), Bbc3 (2.12; P4618), Cradd (2.11; R9775), Cradd (2.01; R5275), Ripk2 (2.06; R9650), Dapk3 (2.18; Z0134), Tnfsf10 (1.83; T9191)
	Both***	Dnase2 (2.08/1.81; D1689)**, Stk17a (1.85/2.23; D1314), Bid (1.89/2.55; B3183), Bbc3 (1.89/2.24; P4743), Bcl-xl (3.14/2.91; B9429)
Cell cycle regulation	EIV A1	Mapk3 (2.24; M7927)
	EIV A2	Mtbp (2.32; M3566), Mapk12 (1.84; M7431), E2f1 (1.9; E9026), TP53 (1.85; P8982)
		Both Cdc27 (2.23/2.4; C7104)
Transcription regulation	EIV A1	– no changes
	EIV A2	Hdac7a (2.19; H2537), Tfap2a (1.95; A9981), c-jun (1.97; J2128), c-jun (2.05; J2253)
	Both	– no changes
Protein quality control	EIV A1	Uchl1(2.12; U5258), Park2 (1.88; P6248)
	EIV A2	Calr (1.86; C4606), Canx (1.9; C4731)
	Both	– no changes
Cytoskeleton system regulation	EIV A1	Ctnnd1 (2.04; P1870), CFL2 (1.96; C8736), Actr3 (2.12; A4721)
	EIV A2	Ptk2 (2.03; F8926), Ptk2b (1.96; P6989), Parva (1.98; A1226), MAPT (1.98; T6819), Ptk2 (1.83; F9051)
	Both	– no changes
Other	EIV A1	Gad2 (1.92; G4913), Gad2 (2.00; G5163), Mapkapk2 (2.33; M3550)
	EIV A2	Rrm2b (1.91; P4993), Sirp-A1 (1.96; S1311), Tnfsf13 (1.99; A7549), Myd88 (2.01; M9934), Tnfsf13 (2.13; A1726)
	Both	– no changes

* – the name of genes referred to the UniProt database (fold change after EIV A1 or EIV A2 infection; Sigma-Aldrich catalog number of antibodies)

** – the name of genes referred to the UniProt database (fold change after EIV A1 infection/ fold change after EIV A2 infection; Sigma-Aldrich catalog number of antibodies)

*** – common proteins up-regulated for both EIV A1 and EIV A2 infection

Discussion

The host-pathogen relationship has enabled viruses to develop a list of various strategies to evade the biochemical and immunological defences of the host. Viruses have acquired the capacity to subvert host cell apoptosis, control of host immune response and inflammation to avoid the immune reactions. Infected cells are eliminated by the programmed cell death and this is the basic defence mechanism against infection. Thus, disabling of the host cell apoptosis may be almost obligatory step in viral life cycle. On the other hand, viruses can profit from stimulating apoptosis to induce the death of the infected cell to disseminate virus progeny. Some of the viral proteins can mimic host-derived apoptosis regulatory proteins like Bcl-2 (Galluzzi et al. 2008). EIV infection of cell causes changes in cell metabolism and stimulates genes showing pro- and anti-apoptotic characteristics. Viral neuraminidase protein (NA) appears to induce apoptosis through indirect and direct mechanisms. Indirectly, NA stimulates transforming growth factor β (TGF-β) *in vivo* and *in vitro* (Schultz-Cherry and Hinshaw 1996, Lowy 2003). Also it was shown that influenza virus NS1 protein induces apoptosis in transfected cells (Schultz-Cherry et al. 2001). Influenza virus causes an increase in p53 protein level – a central player in the tumour suppression. Activated p53 may lead to apoptosis of EIV infected cells (Turpin et al. 2005). Our experiment was designed to characterize changes in the level of expression of proteins in cells infected with EIV A1 and A2 types. We found out up or down-regulation of many proteins (shown in Tables 1, 2) involved in different

Table 2. Proteins (gene names) down-regulated in 3T3 cells – comparison of results obtained in cells inoculated with EIV A1 or EIV A2.

Biological process		Proteins down – regulated in 3T3 cells after EIV infection
Apoptosis	EIV A1	RPS6KB1 (0.8; S4047)*
	EIV A2	– no changes
	Both***	Mapk3 (0.71/0.67; M5670)**
Cell cycle regulation	EIV A1	Cetn1 (0.66; C7736)
	EIV A2	Ccnh (0.71; C5351)
	Both	– no changes
Transcription regulation	EIV A1	Hdac6 (2.11; H2287), Tal (0.79; T1075), Ap1 (0.68; A5968), Smad4 (0.71;S3934)
	EIV A2	– no changes
	Both	Pias2(0.66/0.65; P9498), Gfi1(0.59/0.72; G6670), Ncoa6 (0.74/0.76; A5355)
Protein quality control	EIV A1	BACE1 (0.75; B0806)
	EIV A2	– no changes
	Both	– no changes
Cytoskeleton system regulation	EIV A1	Krt17 (0.78; C9179), Dstn (0.74; D8940), Tuba1(0.76; T9028), Tpm1(0.68; T9283)
	EIV A2	– no changes
	Both	Krt13 (0.73/0.67; C6909), Krt7 (0.79/0.79; C6417), Cald1 (0.64/0.74; C6542), Ppp2ca (0.72/0.75; P8109)
Other	EIV A1	Th (0.73; T2928), RAB9 (0.74; R5404), Erc1 (0.76; E4531), Mta2 (0.75; M7569), Terf1 (0.77; T1948), Ripk1 (0.79; R8274), Scn4a (0.80; S9568)
	EIV A2	Thoc4 (0.81; A9979)
	Both	Gsk3b (0.61/0.59; G7914), Cb2 (0.63/0.68; C5176), Chuk (0.56/0.79; I6139), Spred2 (0.66/0.72; S7320), Hnrnpu (0.76/ 0.74; R6278), Ppp2cb (0.68/0.79; P8609)

* – the name of genes referred to the UniProt database (fold change after EIV A1 or EIV A2 infection; Sigma-Aldrich catalog number of antibodies)

** – the name of genes referred to the UniProt database (fold change after EIV A1 infection/ fold change after EIV A2 infection; Sigma-Aldrich catalog number of antibodies)

*** – common proteins up-regulated for both EIV A1 and EIV A2 infection

cellular processes. We tried to find out how the cells respond to EIV inoculation and what is in common and what differs the strategy of the EIV A1 and EIV A2 multiplication in 3T3 cells. During the multiplication of A1 and A2 different proteins were stimulated. Proteomic approaches like antibody microarrays enable investigating hundreds of proteins in a single experiment. This is the first trial of analysis to look into the complex set of apoptotic proteins stimulated in cells infected with EIV. One marker or one protein out of the apoptotic protein complex does not reflect changes in cells infected with influenza virus. The functional activity of proteins presented in Table 1 and 2 involved in different biological processes overlap. Thus, it is very difficult to point out unambiguously to the function of the particular protein in cells infected with EIV. Our study has revealed that during the infection of cells with influenza viruses there are up or down regulations of many proteins having pro- and anti-apoptotic features, involved in cell cycle regulation, transcription, cytoskeleton regulation and control of proper protein composition and folding (i.e. protein quality).

Four up-regulated proteins found in cells inoculated with either EIV A1 or EIV A2 were: deoxyribonuclease II (gene name: Dnase2), serine/threonine kinase 17a (Stk17a), BH3-interacting domain death agonist (Bid) and BCL2 binding component 3 (Bbc3) belong to the group of apoptosis inducers. Dnase2 is important for DNA fragmentation and degradation during the cell death (Evans and Aguilera 2003). Stk17a is a member of the DAP-kinase-related apoptosis-inducing protein kinase family (Sanjo et al. 1998). Bid, a BCL2 interacting protein, mediates cytochrome C release from mitochondria in response to caspase 8 activated by cell surface death receptors such as FAS and TNF (Luo et al. 1998). In general, BCL2 protein family represents regulators of cellular life-or-death switch and are the central regulator of caspase activation (Levine et al. 2008). Better understanding of how the BCL2 family controls caspase activation during

influenza virus infection can result in new more effective therapeutic approaches. Up regulation of the Bbc3, a strongly pro-apoptotic protein, is a response to diverse apoptotic stimuli. Bbc3 gene expression is activated by at least three apoptotic stimuli, including DNA damage, glucocorticoid treatment, and growth factor deprivation constituting a broad transcriptional response thus far unique among mammalian cell death regulatory genes. Bbc3 induces apoptosis via the mitochondrial apoptotic pathway (Han et al. 2001). In our case it was the infection of cells with influenza virus – either A1 or A2 but precisely how independent cell death signals converge on the regulation of Bbc3 expression in relation with the EIV infection remains to be determined.

Only Bcl-x1 protein represented inhibitors of apoptosis up-regulated in cells inoculated by both A1 or A2 EIVs. This Bcl-2 homologue encodes mitochondrial membrane-associated protein that regulates outer mitochondrial membrane channel (VDAC) opening. VDAC regulates mitochondrial membrane potential, and thus controls the production of reactive oxygen species and release of cytochrome C by mitochondria, both of which are the potent inducers of cell apoptosis (Levine et al. 2008). If the up-regulation of Bcl-x1 is the case in cells infected with influenza viruses, the EIVs could use this mechanism to delay the onset of apoptosis. But this hypothesis needs more experiments to be confirmed. Many authors describe involvement of a particular protein in cell death after inoculation with particular virus. Hepatitis C virus core protein inhibits apoptosis at the mitochondria level through augmentation of Bcl-xl expression resulting in an inhibition of caspase 3 activation (Otsuka et al. 2002). Human T-cell leukemia virus type I tax protein induces the expression of anti-apoptotic gene Bcl-xL in human T-cells (Mori et al. 2001). Miao has shown that hepatitis B virus X protein induces apoptosis in hepatoma cells through inhibiting Bcl-xL expression (Miao et al. 2006). On the other hand, overexpression of Bcl-xL after inoculation of cells with Coxsackie B3 virus delayed the loss of host cell viability and decreased progeny virus release (Carthy et al. 2003).

Bcl2 like protein 11 (Bcl2l 11) is a member of the Bcl2 family with pro apoptotic activity (Levine et al. 2008). It is interesting that some members of Bcl2 family were up-regulated in cells infected with both EIV A1 or A2 (e.g. Bid) but the other dominated in cells infected only with EIV A2 (e.g. Bcl2l11, Bmf). This is the differentiating condition between the multiplication strategy of EIV A2 and EIV A1 in cell cultures 3T3. Obviously both EIVs differ in stimulation of the expression of genes and their products during the multiplication in cell line 3T3. Again this result may indicate why A2 virus is more pathogenic than EIV A1 and still circulates in horse population. Bmf (BCL-2 modifying factor) displays binding affinities to pro-survival Bcl-2 family members, localize preferentially to the outer mitochondrial membrane and induce rapid apoptosis. If so, Bmf blocks the binding of the anti-apoptotic Bcl-2 triggering the cell death (Levine et al. 2008). In general, little is known about the biology of Bmf but the special conserved composition of the protein targets Bmf to the actin cytoskeleton (Grespi et al. 2010). Bmf was up-regulated only in cells EIV A2 infected. Dapk2 (death-associated protein kinase 2) is a protein that belongs to the serine/threonine protein kinase family and its overexpression was shown to induce cell apoptosis. Here we have shown that Dapk2 is up-regulated in EIV A2 infected cells. For comparison this event has not been observed in the cells infected with EIV A1. Consequently, one can suggest that the EIV A2 stimulates stronger pro-apoptotic pathways than EIV A1. In cells Dapk is located to the cytoskeleton in association with the microfilament system (Cohen and Kimchi 2001). Correct localization to the cytoskeleton is crucial for cell killing. Dapk brings together crucial elements of the death machinery and if this protein appears in the cells infected with EIV A2 but not A1 we can carefully assume that the infection of cells with A2 stronger stimulates the cell death than that with A1. As a support of this hypothesis we observed elevated level of Birc5 protein (survivin) after EIV A1 inoculation. The survivin protein function is to inhibit caspase activation, thereby leading to negative regulation of apoptosis. Survivin was reported to prevent apoptosis by binding to caspase-3 in astrocytes infected with Theiler's murine encephalomyelitis virus. Epstein-Barr virus nuclear antigen 1 (EBNA1) confers resistance to apoptosis in EBV-positive B-lymphoma cells through up-regulation of survivin (Lu et al. 2011). Zhang reported up regulation of survivin expression in hepatoma cells stimulated by hepatitis B virus X protein (Zhang et al. 2005).

Influenza virus infection also influences mechanism of cell cycle regulation. It may provide conditions for synthesis and accumulation of viral proteins by inducing G0/G1-phase cell cycle arrest in infected cells (He et al. 2010). In our study, up-regulation of Mapk3 for EIV A1 and E2F1, Mapk12, Tp53 and Mtbp for EIV A2 was observed. Cdc27 protein (up-regulated for both serotypes) as a component of anaphase promoting complex is involved in cell cycle regulation and cell death in response to prolonged mitotic arrest (Lee and Langhans 2012). These proteins play a role in cell cycle progression, reorganization of microtubular system and cytoskeleton. High

levels of some cytoskeletal and microtubular components in cells EIV infected were found (e.g. Cfl2 and Actr3 for EIV A1 but Parva and Mapt for EIV A2).

Among down-regulated proteins (Table 2) some proteins were involved in the regulation of transcription (Pias2, Hnrnup, Ncoa6, Chuk, Ccnh, Thoc4) and cell cycle (Gfl1, Cald1) as well in cytoskeleton and microtubule systems regulation (Cetn1, Tmp1, Dstn, Tuba1). No proteins except Ppp2cb involved directly in the regulation of apoptosis in this group of proteins were found.

In summary, we have found that many proteins up-regulated after EIV infection of cells are involved in the regulation of apoptosis. After EIV A2 inoculation higher levels of stimulators of apoptosis were observed, while after EIV A1 infection both pro- and anti- apoptotic factors were induced. EIV infection promoted changes in the level of proteins involved in the regulation of cell cycle and the rearrangement of the cytoskeleton. We observed changes in the level of these proteins, however different proteins were found to be involved in EIV A1 or A2 infection.

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