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Using the SELDI ProteinChip System to Detect Changes in Protein Expression in Vero Cells after Infection*

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Abstract: Human herpes simplex virus 1 (HSV-1) causes facial, ocular, and encephalitic disease and is associated with latent infection and cancer. Here, we developed a means of studying the pathogenesis of HSV-1 infection at the protein level by using the SELDI Protein Chip to detect changes of protein expression in Vero cells cultured *in vitro*. After infection with HSV-1 and culture for 12, 24 or 48 h, cells were harvested and lysed. IMAC3 arrays were applied to SELDI-TOF-MS to detect proteomic differences before and after infection. The chip detected a series of differentially expressed protein peaks. Interestingly, both peaks at 16 912 Da and 17 581 Da corresponded precisely with the molecular mass of ISG15, which may participate in antiviral activity during the process of infection. Thus, the results we obtained can serve as a basis to study the pathogenesis of HSV-1 and the interaction between the virus and its host. In addition, they can help in the discovery of new therapeutic targets for treatment of HSV-1 infection.

Key words: SELDI Protein Chip; Vero Cells; HSV-1; Protein Expression

Herpes simplex virus (HSV) belongs to the *Herpesviridae* family. HSV-1 and HSV-2 are differentiated in terms of method of transmission, as well as epidemiology, clinical manifestations, and biochemical/biological characteristics. HSV-1 is normally associated with orofacial infections and encephalitis, whereas HSV-2 usually causes genital infections and can be transmitted from infected mothers to infants(6). Both viruses establish latent infections in sensory neurons and upon reactivation both cause lesions at or near the point of entry into the body. The transmission pathways of HSV-1 are classified as *via* the respiratory tract, skin, mucous membranes and close contact. Skin, mucous membranes and organs above the waist are easily infected. According to an epidemiological investigation, HSV-1 is the cause in about 70% of sporadic encephalitis cases (18). Multiple organs are involved after the HSV-1 infection and the correlated diseases take a long time to heal. While most of the virus is eliminated as the body develops immunity, some remains in trigeminal ganglion cells or astroglia, persistent and latent in the host without causing clinical symptoms. When the host is

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immunodepressed, as with certain bacterial or viral infections, the latent virus is activated to proliferate. Furthermore, HSV-1 is associated with esophageal carcinoma, oral cavity squamous cancer, cheilocarcinoma, carcinoma vulvae and cervical cancer (12, 19, 21).

The processes by which viruses infect cells are complex. On the one hand, the existence and proliferation of the virus depends on its host cell, in which apoptosis is suppressed to permit replication. On the other hand, organisms initiate apoptosis to eliminate infected cells (1). Many viral mothers to infants(6). Both viruses establish latent infections in sensory neurons and upon reactivation both cause lesions at or near the point of entry into the body. The transmission pathways of HSV-1 are classified as via the respiratory tract, skin, mucous membranes and close contact. Skin, mucous membranes and organs above the waist are easily infected. According to an epidemiological investigation, HSV-1 is the cause in about 70% of sporadic encephalitis cases (18). Multiple organs are involved after the HSV-1 infection and the correlated diseases take a long time to heal. While most of the virus is eliminated as the body develops immunity, some remains in trigeminal ganglion cells or astroglia, persistent and latent in the host without causing clinical symptoms. When the host is immunodepressed, as with certain bacterial or viral infections, the latent virus is activated to proliferate. Furthermore, HSV-1 is associated with esophageal carcinoma, oral cavity squamous cancer, cheilocarcinoma, carcinoma vulvae and cervical cancer (12, 19, 21).

The processes by which viruses infect cells are complex. On the one hand, the existence and proliferation of the virus depends on its host cell, in which apoptosis is suppressed to permit replication. On the other hand, organisms initiate apoptosis to eliminate infected cells (1). Many viral infections therefore exhibit apoptosis and anti-apoptosis. Similarly, infections caused by HSV-1 either promote or resist apoptosis (2,3,14,15,20).

In this experiment, we cultured Vero cells conventionally in vitro. After infection with HSV-1 and culture for different times (12, 24 or 48 h), cells were harvested and disrupted with lysis solution. IMAC3 arrays were applied to SELDI-TOF-MS to detect the proteomic differences before and after infection. The chip detected a series of differentially expressed protein peaks. The results establish a basis for studying the pathogenesis of HSV-1 infection at the protein level and the interaction between HSV-1 and host cells, as well as in searching for target sites for anti-infection therapy

1. Materials and methods

1.1 Cells and viruses

Vero (African green monkey kidney) cells were grown in Dulbecco′s modified Eagle′s medium supplemented with 5% fetal calf serum. The HSV-1 wild-type strain was propagated, and its titer was determined in Vero cells. The HSV-1(SM44 strain)from the institute of virology, Chinese Academy of Preventing Medicine, was propagated in Veros cells. Vero cell monlayers at about 90% confluence were infected with HSV-1 at a multiplicity of infectiom (MOI) of 0.5 PFU per cell).

1.2 Cell harvest and extraction of total proteins

Cells infected with HSV-1 were harvested after 12, 24 or 48h. Normal Vero cells were harvested at the same times. Cells were scraped off with a sterile scraper and washed three times in pre-cooled phosphate buffer solution, followed by cell counting. Lysis solution (8 mol/L Urea, 4% CHAPS, 40 mmol/L Tris-HCl, pH7.4) was added, 200 μL for 5×106 cells. Lysate was shaken intensely for 30 min at 4℃ and centrifuged for 30 min at 14 000

r/min. A protein-nucleic acid analyzer was used to ass ay the concentration of proteins in the supernatant. All sample concentrations were adjusted to 1.5 mg/mL by adding lysis solution. Supernatant was stored at -80℃.

1.3 The IMAC3 ProteinChip array protocol

The array protocol followed the supplier′s instructions (ProteinChip Applications Guide, Ciphergen). 5 μL (50 mmol/L) nickel sulfate was applied to each spot and incubated in a humidity chamber for 15 min, then the procedure was repeated. Excess nickel was removed by rinsing in deionized water. 5 μL 0.5 mol/L NaCl in PBS was applied to each spot and incubated on a shaker for 5 min, then 200 μL binding buffer was added to each well. After incubation for 5 min at room temperature with vigorous shaking, the buffer was removed from the wells and 50 μL/well samples diluted in binding buffer were immediately added. After 30 min incubation, washing and air-drying, 0.5 μL SPA was applied to each spot, air-dried, and analyzed in the ProteinChip Reader (Cliphergen Biosystem, Inc, Fremont, CA).

1.4 Reproducibility

Cells were harvested three times to control for inter-group differences. Each sample was detected by IMAC3 and WCX2. Detection was performed repeatedly on the same arrays to control for disparities between arrays. The same samples were taken and tested a week later to verify reproducibility.

1.5 Data Acquisition and Analysis

Data were read by the ProteinChip Biosystem (PBSIIC) in protein arrays. The instrument was calibrated with standard polypeptides. The system′s mass deviation was 0.1%. The parameters were set as follows: laser intensity 210, detection sensitivity 9, optimized molecular mass range 2.0~00-10.0 k Da and maximum molecular weight 50.0k Da.Data were collected automatically by the analysis software (Ciphergen ProteinChip Version 3.0) and protein mass spectrogram differences between the two types of cells were analyzed by Biomarker Wizard Software.

2. Results

2.1 Morphological Changes in Cells after Viral Infection

Cell morphology appeared to be grossly normal in the first 12 h after HSV-1 infection. By 24 h, cytopathological effects were evident in more than 50% of cells, seen as cellular swelling, cellular confluence and a honeycomb appearance. After 48 h of infection, cytopathological effects were present in almost 100% of cells, with honeycomb-giant cells, and some cells shedding (Fig.1).

2.2 Reproducibility

The reproducibility showed that protein peaks captured from different batches, from the same sample with

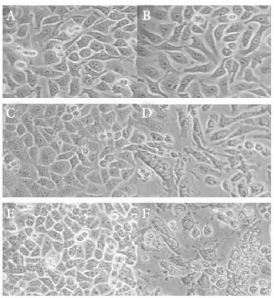

Fig. 1. Cell morphology before and after HSV-1 infection. A: Non-infected Vero cells at 12 h; B: Vero cells infected by HSV-1 for 12 h; C: Non-infected Vero cells at 24 h; D: Vero cells infected by HSV-1 for 24 h; E: Non-infected Vero cells at 48 h; F: Vero cells infected by HSV-1 for 48 h.

different arrays and for different times were equivalent (Fig. 2). Within the molecular weight range from 2 000 to 30 000 Da,IMAC3 captured 58 protein peaks. Nine different protein peaks were found to change their expression levels after infection. Expression of proteins 10.14, 11.28, 16.91, 17.05, 17.22 and 17.57 kDa in size increased after infection, while that of proteins of 6.28, 6.65 and 18.91 kDa was lower.

2.3 Initial identification of candidate proteins

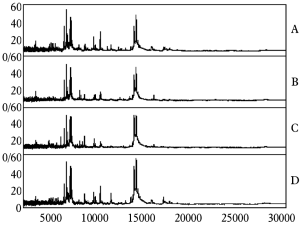

Fig. 2. Protein peaks at 2 000-30 000 Da detected by IMAC3. Panels A and B show the same sample at different times; protein concentration in both was 2 mg/mL. Panels C and A show the same sample detected by different arrays. Panel D shows the same sample as in panel A, but collected a week later.

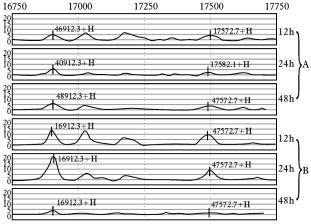

Fig.3. The proteins of 16.91 kDa and 17.57 kDa were only high at 12 and 24 h after infection. The peaks were maximal at 24 h and were depressed at 48 h.A:Non-infected cells.B:Infected cells.

The molecular weights and isoelectric points were input into the Swiss protein data base (http://cn.expasy.org/tools/tagident.html) to search on the basis of proteins captured by the IMAC3 (pI 7.2) arrays. We found the protein peak at 16. 91 kDa and 17.58 kDa was close to interferon-stimulated gene 15 (ISG15; pI 7.03, MW 17.01 k Da and 17. 76 kDa). We did not find any other proteins matching the peaks, and presume these are new proteins.

3. Discussion

The proteome is defined as the entire complement of proteins expressed by a cell, organism, or tissue type, and accordingly, proteomics is the study of this complement expressed at a given time or under certain environmental conditions(17). Surface Enhanced Laser Desorption/Ionization Time of Flight Mass Spectrometry (SELDI-TOF-MS) is a proteomics technology developed in recent years. It can detect proteins or polypeptides ranging between 500 and 500 000 Da in very small samples (0.5-400 μL), as well as proteins present at the level of femtoliters (4,7) .

Recent studies indicate that HSV-1 induces a primary gene response in cells, which generates specific gene products, such as TIS7(13). Li found that proto-oncogenes, including c-fos, src and ras are differentially expresse d after infection with HSV-1. By applying 2-D gel electrophoresis and MALDI-TOF-MS to serum from HSV-1 infected patients(9,5), found changes not only in the expression of proteins, but also in their characteristics(5).

In this experiment, we applied SELDI-TOF-MS, intending to find pathogenic protein markers related to HSV-1 infection and then to reveal the pathopoietic mechanisms underlying diseases caused by HSV-1. We divided the Vero cells into experimental and control

groups to detect differences in the supernatant of the lysate and found inflammatory factors that may be related to viral infection and morbidity after comparing the protein spectra with candidate proteins in the Swiss protein database.

Controls for inter-group differences and disparities between arrays showed that our experiments had good reproducibility and high sensitivity. After 24 h of infection with HSV-1, obvious cytopathic effects appeared in the Vero cells. After 12 h of infection, differential expression was seen by mass chromatographic analysis, implying that proteome changes preceded those of morphology. The IMAC3 array revealed a series of 9 differentially expressed protein peaks. Intracellular proteins are known to change in their components and quantities leading to viral diseases. Some of these changes can be detected from serum proteins. In a previous study using differential expression analysis of the serum from HBV carriers, hepatocellular carcinoma patients and normal controls, we found 18 differentially expressed peaks. Compared with the sera, in the present study we found the same protein both at 16 912 and 17 581 Da may be interferon-stimulated gene 15 (ISG15), which was maximal at 24 h and depressed at 48 h. This may be a tissue-specific protein secreted by infected cells into the serum.

In controlling viral infections, the alpha/beta interferon (IFN-α/β) system acts as an important component of the response. IFN-α/β are expressed in response to viral infection and exert their effects through the IFN-α/β receptor, resulting in the activation of JAK/STAT-dependent and STAT-independent pathways and the subsequent induction of hundreds of genes containing interferon-stimulated response elements (ISREs)(16). The interferon-stimulated genes (ISGs) have been shown to directly inhibit viral replication (11). ISG15 is one of the earliest ISGs induced following IFN stimulation. It is expressed in a variety of cell types at low levels and induced within 6 h of stimulation with IFN-α/β, and to a lesser degree by IFN-γ(10). Lenschow demonstrated that this protein has antiviral activity(8). It seems that ISG15 can reduce the fatality rate and depress viral replication in multiple organs, but cannot suppress the spread of viruses in the host. Therefore, the increased level of ISG15 in our experimental cells may reflect immunoprotection when cells have been removed for their inherent immunity. In the later period of viral infection, when HSV-1 completed DNA replication, nucleocapsid packaging and virus maturation and release, it inhibited the host Vero cells′ normal physiological functions and led to cytopathological effects, seen under the microscope after 48h. The downregulation of ISG15 in the post-infection period may explain the functional incapacitation and structural failure of host cells. The protein peaks we found at 16 912 and 17 581 Da may have been the ISG15 protein. However, since this was only on the basis of molecular weight, further investigations are needed for definitive identification.

To sum up, we found that cytokines are closely related to the HSV-1 infection of Vero cells. Further assays and analyses are needed to verify our suggestions. Thus, the results contribute to the study of the effects of the pathopoiesis of HSV-1 on protein levels, exploring the interactions between HSV-1 and host cells, and searching for anti-infection therapy targets. Our next aim is to study and identify these differential peaks, to further understand viral infection as well as viral interactions with host cells.

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