H5N1 Virus Causes Significant Perturbations in Host Proteome Very Early in Influenza Virus-Infected Primary Human Monocyte-Derived Macrophages

Chung Yan Cheung,1 Eric Y. Chan,2,4 Alexei Krasnoselsky,2 David Purdy,2 Arti T. Navare,2 Janine T. Bryan,2 Carolina K. L. Leung,1 Kenrie P. Y. Hui,1 Joseph Srijal Malik Peiris,1 and Michael G. Katze2,3

1Centre of Influenza Research & School of Public Health, University of Hong Kong; 2Department of Microbiology, School of Medicine, and 3Washington National Primate Research Center, University of Washington, Seattle

H5N1 influenza viruses, which cause disease in humans, have unusually high pathogenicity. The temporal response of primary human monocyte-derived macrophages infected with highly pathogenic H5N1 and seasonal H1N1 influenza viruses was evaluated using mass spectrometry–based quantitative proteomic profiling. This was done in order to demonstrate significant perturbation of the host proteome upon viral infection, as early as 1 hour after infection. This early host response distinguished H5N1 infection from H1N1 infection, the latter inducing less of a response. The most pronounced effect was observed on the translational machinery, suggesting that H5N1 might gain advantage in replication by using the cell protein synthesis machinery early in the infection.

Influenza virus subtype H5N1, which remains entrenched in poultry flocks in many countries, occasionally infects humans, causing disease with unusually high morbidity and mortality. Transmission between humans does occur but fortunately is inefficient. Current estimates of a case fatality rate of >50% associated with confirmed H5N1 disease may be inflated due to a case-ascertainment bias. However, it is clear that the pathogenesis of human H5N1 disease is fundamentally different from that caused by more typical human seasonal influenza viruses [1].

High-throughput transcriptomic studies on the host response to H5N1 viruses have been reported in a variety of in vivo animal models [2–4]. Transcriptomic analysis of the global host response profile of primary human macrophages infected in vitro with comparable virus input has demonstrated major differences between H5N1 and seasonal influenza H1N1 viruses [5], which are similar to those observed in vivo. At the protein level, the virus-induced changes in the host do not necessarily follow closely the corresponding gene expression [6], yet they might reveal critical host response to pathogens.

To the best of our knowledge there have not been reports comparing the differences in the cellular host response between H5N1 and H1N1 viruses at the proteomic level, especially in the early phase of infection. As macrophages are key orchestrators of the immune response, primary human monocyte-derived macrophages (MDMs) were infected with H5N1 or H1N1 influenza viruses in this study, and quantitative high-throughput proteomic analysis was applied to investigate the change in host proteome after infection with these influenza virus strains.

MATERIALS AND METHODS

Primary Monocyte-Derived Macrophages
Peripheral blood leukocytes were separated from buffy coat obtained from healthy blood donors (Hong Kong Red Cross Blood Transfusion Service). Then monocytes were isolated by plastic adherence and allowed to differentiate into macrophages for 14 days in vitro as previously described [7].

Viruses and Infection
A human H1N1 virus isolated from a patient with mild seasonal influenza (A/Hong Kong/54/98 [H1N1]) was used to represent a low pathogenic influenza virus. A virus from a patient with fatal H5N1 disease in Vietnam (A/Vietnam/3212/04 [3212/04] [H5N1]) was used to represent a highly pathogenic virus for comparison. Influenza viruses were propagated in Madin Darby Canine Kidney cells as described previously [8]. Viruses were concentrated and purified by filtration through 100-kDa filtration units (Millipore) to remove cellular...
mediators that may confound the analysis. After resuspension of the viruses in fresh culture medium, the infectivity of the virus stock was determined by Tissue Culture Infective Dose 50% (TCID50) titration on MDCK cells and by infectious center assay using MDMs as previously described [9]. Macrophages were infected at a multiplicity of infection of 2, and cellular proteins were extracted from cells at 1, 3, and 6 hours post infection (p.i.) as described previously [8].

Mass Spectrometry Sample Preparation, Data Acquisition, and Analysis

Protein extracts were prepared and analyzed using liquid chromatography-mass spectrometry (LC-MS) analysis as described previously [10]. Global protein quantification was performed based on the MS signal intensities of constituent tryptic peptides, with protein identities derived from data-dependent LC-MS/MS searched against the human International Protein index (IPI) database (version 3.64) and filtered by X!Tandem with a log(E value) ≤ −5. Analysis was carried out on 4 independent experiments, each with different donors.

Statistical and Functional Analysis

All data processing and statistical analysis were performed in Matlab using Statistical Toolbox and custom scripts. The raw intensity data were normalized by Elucidator and combined dataset further quantile normalized. Principal component analysis (PCA) was performed on log_{10} transformed normalized peptide intensities. The peptide ratios were produced relative to the median of the time-matched mocks to reduce experimental variation. Because the analysis described above was performed on the peptide level, we further filtered the signature proteins to reduce the number of spurious hits. The proteins were filtered by the overall concordance, requiring that at least half of the peptides follow the same directionality. Protein ratios were computed from peptide ratios greater than 1.4. Furthermore, only the peptides concordant between all 4 biological replicates were selected for protein ratio rollup. The significantly up- or downregulated proteins were selected by further requiring concordance between more than 50% of observed peptides. Differentially abundant proteins were subjected to Gene Ontology (GO) and pathway enrichment analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID), Ingenuity Pathways Analysis (IPA; Ingenuity Systems), and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING)-known and predicted protein-protein interactions analysis STRING database, version 9.0) [11].

RESULTS

H5N1 Induced Significant Perturbation of the Proteome at Very Early Stages of Viral Infection

We quantified changes in the abundance of 1648 proteins that occurred at 1 hour, 3 hours, and 6 hours p.i. in H1N1- and H5N1-infected MDMs as well as time-matched mock-infected samples. When the data were analyzed separately for each time point by PCA, the proteomic profile of H5N1-infected macrophages at 1 hour p.i. showed a clear difference from both H1N1- and mock-infected cells.

We selected peptides that showed more than a 1.4-fold change compared with mock infected cells, retaining those peptides that showed concordance across all 4 MDM donors (biological replicates). We observed that the PCA of these peptides clearly showed the predominant separation of H5N1 1 hour p.i. from the rest of the samples. To select peptides that more precisely reflected the signature of H5N1, we only included the peptides that contributed to this PCA separation (ie, laying closer to the 3d principal component). The resulting signature consisted of 1146 peptides corresponding to 247

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Table 1. DE\* Proteins Identified in H5N1- or H1N1-Infected Monocyte-Derived Macrophages

<table>
<thead>
<tr>
<th>Virus</th>
<th>Hours Postinfection</th>
<th>All DE Proteins</th>
<th>DE Proteins Identified With ≥2 Peptides</th>
<th>All DE Proteins</th>
<th>DE Proteins Identified With ≥2 Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5N1</td>
<td>1</td>
<td>181</td>
<td>119</td>
<td>150</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>94</td>
<td>60</td>
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<tr>
<td></td>
<td>6</td>
<td>164</td>
<td>102</td>
<td>108</td>
<td>65</td>
</tr>
<tr>
<td>H1N1</td>
<td>1</td>
<td>51</td>
<td>26</td>
<td>32</td>
<td>13</td>
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<td></td>
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<td></td>
<td>6</td>
<td>65</td>
<td>33</td>
<td>56</td>
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Abbreviation: DE, differentially expressed.

* DE proteins were defined to have more than half of the peptides showing fold changes greater than 1.4 in the corresponding direction as compared to the time-matched mock-infected cells (see Results section for details).
upregulated proteins. Similar selection of downregulated proteins resulted in 1045 peptides corresponding to 468 proteins. In contrast with the significant perturbations of host proteome at 1 hour p.i. with H5N1 infection, significantly smaller differences were detected at 3 hours p.i., yet the clear separation between H5N1- and H1N1-infected cells remained in PCA analysis. Table 1 summarizes the final numbers for up- and downregulated proteins for H5N1- or H1N1-infected MDMs. Comparison of the differentially regulated proteins across all 3 time points for the same virus revealed that the protein changes that occur early after H5N1 infection are generally not sustained for longer than 6 hours p.i. The virus infection-associated changes were significantly less pronounced for H1N1 in terms of the number of proteins changing in abundance (Table 1). The functional analysis (see following section) revealed that early response of MDM to H5N1 was very different from the response to H1N1.

Functional Analysis of Host Proteomic Profiles During H1N1 and H5N1 Infections

The filtered signature proteins served as input for the functional analyses by DAVID, STRING, and IPA. The enrichment analysis was performed on differentially regulated proteins for each virus infection at 3 time points. The top most significantly enriched category by DAVID enrichment analysis for H5N1-infected MDMs at 1 hour p.i. was translation ($P < 4 \times 10^{-4}$). IPA analysis of this category of proteins showed that not only ribosomal proteins but elongation factors as well were significantly affected in H5N1-infected MDMs compared with mock-infected cells (Figure 1A and 1B). Interestingly, these changes became less pronounced at 3 hours p.i. and 6 hours p.i. The perturbations of the translational machinery in H5N1-infected MDMs were still significant at 3 hours p.i. as well. In contrast, no such effect was detected in H1N1-infected MDMs at 1 hour p.i. or later. However, IPA analysis showed that 3 ribosomal proteins (RPS25, RPS18, and RPS5) were modulated with similar profiles across both viruses during the time of infection (Figure 1C). Validation of the protein changes for RPS18, RPL14, and RPS5 by Western blot analysis did not result in detection of all of these proteins. Only RPS18 was successfully detected by Western blot, possibly reflecting the variation in antibody sensitivity and differences in cellular expression. In 3 independent Western blot analyses we found that the abundance of RPS18 in H1N1-infected MDM increased by 1.4 ± 0.11 over mock infected cells; whereas in H5N1-infected MDM, abundance increased by 1.6 ± 0.15 (Figure 2C). The findings were true for RPS18 in infected MDMs as well as A549 cells.

IPA network analysis also revealed the upregulation of vacuolar ATPase (vATPase) complex proteins and the protein abundance of constituents of the vATPases network increased as early as 1 hour p.i. for both H5N1 and H1N1 viruses. Throughout the 6 hours after infection, H5N1-infected MDMs maintained this elevated level of proteins in the vATPase network, while the levels gradually decreased in H1N1-infected MDMs. Among downregulated proteins, lysosome and exopeptidase activity ($P < .025$), endosome to lysosome transport ($P < 2.6 \times 10^{-4}$), and metalloproteases ($P < .029$) were also enriched, suggesting possible rapid secretion of these proteins. In addition, cell death ($P < .017$) and apoptosis ($P < .037$) pathways were moderately enriched among downregulated proteins.

At 6 hours p.i., none of the pathways or biological processes reached the level of significant enrichment for protein changes in both H5N1- and H1N1-infected MDMs. However, proteins associated with antigen processing and presentation of exogenous antigen ($P < .003$) reached the level of significance in H5N1-infected MDMs.

DISCUSSION

Our proteomic analysis revealed that MDMs infected by the highly pathogenic H5N1 influenza virus as early as 1 hour p.i. respond to the viral infection with a dramatic perturbation in protein abundance in components associated with protein synthesis machinery. The most prominent early response (1 hour p.i.) was marked by the change in abundance of ribosomal proteins and elongation factors. The levels of ribosomal proteins such as RPS25, RPS18, and RPS5 were modulated after infection by both influenza viruses. However, it is important to note that RPS25 and RPS18 were significantly upregulated at 1 hour and 3 hours p.i. in H5N1 macrophages. These 3 proteins comprise a part of the small 40S subunit and are in close physical proximity on the ribosome. Moreover, RPS25 has been implicated as playing a crucial role in the cap-independent translation initiation mediated by IRES RNA present in the intergenic region of several viruses of the family Dicistroviridae [12]. Although influenza viruses are thought to initiate replication by a cap-snatching mechanism, the question of whether the H5N1 virus has harnessed the additional ability to use the cap-independent mode of translation initiation is intriguing but remains to be explored. We observed at 6 hours p.i. that both H5N1- and H1N1-infected MDMs displayed a decrease in abundance of proteins with the elongation factors network, most notably eukaryotic initiation factor 2 (EIF2), which is involved in the initiation of translation. Degradation of host mRNA and protein synthesis shut-off have been previously described at 4 hours to 6 hours p.i. for influenza viruses [13]. Further work to elucidate the molecular mechanism through which influenza viruses interact with EIF2 and the proteins within the elongation factor network is warranted.

We have previously observed that H5N1 induces a potent pro-inflammatory response in MDMs, which is the result of...
Figure 1. Analysis of the differentially abundant proteins using Ingenuity Pathways Analysis (IPA). Protein synthesis, molecular transport, and nucleic acid metabolism networks as defined by IPA. A, The network modulation is shown for H5N1-infected monocyte-derived macrophages (MDMs) at 1 hour p.i. The blue circles identify double-stranded DNA break repair proteins. Blue squares identify proteins associated with virus infection. The red circle defines a group associated with molecular transport and nucleic acid metabolism. B, Protein synthesis machinery proteins differentially expressed at 1 hour p.i. in H5N1-infected MDMs. Blue squares identify proteins that belong to the protein elongation functional group in GO. C, Time-course changes in protein abundance in both H1N1- and H5N1-infected MDMs for the network described in part A.
Our assessment of the change in the protein landscape in macrophages after H5N1 and H1N1 infection has provided a road map for further investigation into the role of the protein translational network, as well as other affected cellular networks or proteins during influenza virus infection revealed in this study. Such investigation will further our understanding of the molecular mechanisms that determine influenza pathogenicity.

Notes

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References