Journal of Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Quantitative proteomics identify an association between extracellular matrix degradation and immunopathology of genotype VII Newcastle disease virus in the spleen in chickens

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ARTICLE INFO

Keywords:
Newcastle disease virus
Genotype VII
Extracellular matrix
MMP
Immunopathology
Chicken spleen

ABSTRACT

Pathogenesis of genotype VII Newcastle disease virus (NDV) is characterized with remarkable immunopathology in the spleen in chickens. However, the mechanism for this unique pathological phenotype is not fully understood. Previous transcriptomics data showed that genotype VII NDV JS5/05 caused a greater downregulation of extracellular matrix (ECM) genes than genotype IV virus Herts/33 in the spleen. In this study, the role of ECM in pathology of genotype VII NDV was investigated using quantitative proteomics. Pathology studies showed that JS5/05 caused severe immunopathology characterized with remarkable necrosis in the spleen, whereas Herts/33 only induced mild pathological changes. The ECM was firstly enriched from the spleens and ECM proteins of different categories were identified by LC-MS/MS. Quantitative proteomic analysis showed that JS5/05 caused a significant disruption of ECM integrity and molecular composition compared to Herts/33. Particularly, JS5/05 induced a more remarkable collagen breakdown in the spleen compared to Herts/33. Moreover, matrix metalloproteinase (MMP)-13 and -14 were significantly upregulated by JS5/05 infection. KEGG pathway analysis suggested that differential regulation of ECM proteins by JS5/05 and Herts/33 may impact pathology through different pathways. Therefore, our results suggested that MMP upregulation and consequent ECM degradation contribute to immunopathology of genotype VII NDV in the spleen.

Significance: Pathogenesis of genotype VII NDV is characterized with severe immunopathology in the spleen in chickens. Elucidating the mechanism of this pathology phenotype is critical to understand pathogenesis of genotype VII NDV. Here, we present the proteomic data of an important non-cellular compartment, the extracellular matrix (ECM), in the spleen from chickens infected with genotype VII and IV NDVs. Our results suggest that significant upregulation of matrix metalloproteinases by genotype VII NDV and consequent disruption of ECM integrity and composition may be associated with immunopathology in the spleen. Moreover, ECM degradation, represented by collagen breakdown, is an important pathology event in the process of genotype VII NDV infection. Our study for the first time presents evidence of ECM regulation by NDV and adds ECM remodeling as a new manifestation for NDV pathology. Our findings also deepen the understanding of NDV pathogenesis.

1. Introduction

Newcastle disease (ND) is one of the most important infectious diseases for poultry. Newcastle disease virus (NDV), the causative agent of ND, has only one serotype, whereas genotypic diversity and rapid evolution of the virus still pose challenges for disease control [1,2]. Genotype VII NDV has emerged in late 1980s in the Far East and is classified as the late genotype (after 1960s) [3], which is currently dominant in many countries worldwide [4–6]. Pathogenesis of genotype VII NDV is characterized with remarkable immunopathology in the

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https://doi.org/10.1016/j.jprot.2018.04.019
Received 22 November 2017; Received in revised form 2 April 2018; Accepted 17 April 2018

spleen compared to viruses of the early genotype (e.g genotype IV) [7–9].

The aberrant innate immune response, also termed as “cytokine storm”, contributes to immunopathology caused by genotype VII NDV in the spleen. Ecco et al. reported that genotype VII NDV strain ZJ1 induces a stronger innate immune response in the spleen compared to NDV strains belonging to other genotypes [10]. Hu et al. have shown that two genotype VII NDV strains elicit a more potent cytokine response in the spleen compared to strains of the early genotype (IV and IX), which is associated with the severe splenic necrosis caused by genotype VII viruses [8]. In addition, two independent studies have revealed that genotype VII NDV also acts as a hyperinducer of the cytokine response in the immune organs of waterfowls (geese and ducks) [11,12]. An intense cytokine response was thought to be related to high levels of virus replication of genotype VII NDV in the spleen. However, the mechanism of immunopathology of genotype VII NDV in the spleen is still not fully understood.

We have previously demonstrated that genotype VII NDV significantly upregulated expression of innate immune-related genes in the spleen compared to genotype IV virus using the transcriptomics technology [8]. Datamining of the transcriptomics data showed that genotype VII and IV viruses also greatly differ in regulating extracellular matrix (ECM) genes in the spleen of chickens. Viruses of both genotypes downregulated expression of most ECM genes, whereas degree of downregulation induced by genotype VII strain was much greater compared to genotype IV NDV (unpublished data). Therefore, we hypothesized that changes in the ECM may be associated with immunopathology of genotype VII NDV in the spleen.

The ECM is the non-cellular component present within all tissues and organs and is a complex of meshwork composed of highly cross-linked proteins. The ECM provides not only essential biophysical support and anchorage for the cellular constitutes but also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, homeostasis, cell proliferation, and migration [13–15]. The ECM is also a major component of the tumor microenvironment and actively regulates tumor metastasis and progression [16,17]. Moreover, ECM remodeling also affects pathologies of different pathogens. Talmi-Frank et al. have shown that H1N1 influenza virus infection activates matrix metalloproteinase (MMP)-14, a proteinase for ECM degradation, in mouse lung, causing subsequent proteolysis of the ECM, which contributes to the severe tissue damage in the lung and mortality of mice [18]. In addition, AI Shammari et al. have demonstrated that *M. tuberculosis* induces destruction of collagen, a key component of the ECM, which subsequently results in cell death of mononuclear cells and contributes to immunopathology and granuloma necrosis in tuberculosis (TB) [19]. The presence and distribution of ECM components have also been identified in chickens [20]. ECM destruction in the antigen-trapping zone in the spleen leads to impairment of tissue organization, which may contribute to the permanent immunosuppression caused by infectious bursal disease virus [21]. More importantly, ECM constitutes, notably heparin sulfate and collagen, were found to limit NDV spread in solid tumor tissues and diminish the potential oncolytic activity of the virus [22]. Therefore, the ECM is a key regulator of pathogenesis of different pathogens by affecting tissue integrity, cell death or the inflammatory response.

In the present study, we aim to characterize the role of the ECM in immunopathology caused by genotype VII NDV in chicken spleens. We first enriched ECM proteins from the spleens of chickens infected with genotype VII and IV NDVs using an established decellularization procedure. Abundance of ECM proteins were then determined using tandem mass tags (TMT)-labeled quantitative proteomics. Proteomic data were analyzed using multiple bioinformatics systems and validated by ELISA and ECM staining. Moreover, expression of representative collagenases (MMP-1, -13 and -14) following NDV infection was profiled using quantitative Real-time PCR (qRT-PCR) and immunoblotting. Genotype VII NDV strain JS5/05 and genotype IV strain Herts/33 were selected because this pair of viruses were used in our previous transcriptomics study, and they differ greatly in the pathological manifestation, the innate immune response profile and regulation of ECM genes. Our results suggested that genotype VII NDV significantly activated MMPs and induced a remarkable ECM degradation in chicken spleens compared to genotype IV NDV, which may be associated with immunopathology phenotype.

2. Materials and methods

2.1. Ethics statements

All animal experiments were approved by the Jiangsu Administrative Committee for Laboratory Animals (Permission number: SYXK-SU-2007-0005), and complied with the guidelines of Jiangsu laboratory animal welfare and ethics of Jiangsu Administrative Committee of Laboratory Animals. All experiments involving live virulent NDVs were performed in animal biosecurity level-3 facilities.

2.2. Viruses and cells

Genotype VII NDV strain JS5/05 and genotype IV NDV strain Herts/33 are typical velogenic strains. Information for these two strains has been provided elsewhere [8,9]. Virus titers were measured as 50% embryo infectious dose (EID_{50}) in 9–11-day old specific-pathogen-free (SPF) embryonated chicken eggs using the Reed and Muench method [23]. Chicken embryo fibroblasts (CEFs) were maintained in M199 medium supplemented with 4% fetal calf serum.

2.3. Chicken infection study

Ten 4-week-old SPF chickens were intranasally (i.n.) inoculated with 10^5 EID_{50} of JS5/05 or Herts/33 in 0.1 mL. Another ten chickens were inoculated with PBS as the sham control. Chickens were daily monitored for clinical symptoms. At day 1 and 3 post-inoculation (pi), three chickens per group were euthanized for gross pathology observation and tissue collection. Gross lesions in the spleen were scored based on the standards reported elsewhere [8]. Spleens were processed for virus load measurement, histological assessment and ECM protein enrichment. Histopathological changes of the spleens were scored according to the criteria as described previously [8]. Remaining four birds per group were monitored for mortality and clinical signs.

2.4. Virus load measurement

Spleens were homogenized in PBS, and virus titers were measured in CEFs. Briefly, the cleared tissue homogenates were serially 10-fold diluted and inoculated in CEF. At 72 h pi, cytopathic effect in cells inoculated with each dilution was observed. Virus titer was calculated as 50% tissue culture infectious dose ($TCID_{50}$) per gram using the Reed and Muench method [23]. Virus load of JS5/05 and Herts/33 groups were statistically compared using *t*-test, and $p < .05$ was considered as a significant difference.

2.5. ECM protein enrichment and monitoring

The insolubility of ECM proteins has hindered the purification and systematic characterization of the composition of in vivo extracellular matrices of tissues. A decellularization procedure that results in the extraction (or depletion) of cytosolic, nuclear, membrane and cytoskeletal proteins and the enrichment of ECM proteins has been developed [24]. Sequential extractions of the frozen spleen tissues were performed using the CNMCS (**C**ytosol/**N**ucleus/**M**embrane/**C**yto**S**keleton) Compartmental Protein Extraction Kit (Millipore, Temecula, CA, USA) as described previously [24]. Three independent spleen samples were taken from JS5/-5-, Herts/33- and mock-infected chickens at day