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Short Communication

Immune characterization of long pentraxin 3 in pigs infected with influenza virus

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ABSTRACT

Long pentraxin 3 (PTX3) is a conserved pattern-recognition secreted protein and a host-defence-related component of the humoral innate immune system. The aim of the present study was to characterize swine PTX3 (SwPTX3) protein expression in influenza virus infected pigs. First, we performed *in silico* studies to evaluate the cross-reactivity of PTX3 human antibodies against SwPTX3. Secondly, we used *in vitro* analysis to detect SwPTX3 presence in swine bone marrow dendritic cells (SwBMDC) upon stimulation with different agents by Western blot and immunofluorescence. Finally, the levels of SwPTX3 were assessed in experimental infection of pigs with different strains of influenza virus. This is a novel study where the expression of SwPTX3 was evaluated in the context of a pathogen infection. The initial characterization of SwPTX3 in influenza virus infected pigs contributes to understand the role of PTX proteins in the immune response.

1. Introduction

The innate immune system constitutes the first line of defence against microorganisms and plays a primordial role in the activation and regulation of adaptive immunity. Components of the humoral arm include members of the complement cascade and soluble pattern recognition molecules (PRMs), such as collectins, ficolins and pentraxins (Bottazzi et al., 2010; Deban et al., 2011). Pentraxins constitute a superfamily of acute phase multifunctional proteins with multimeric organization that are phylogenetically conserved from arachnids to mammals representing the prototypic component of humoral innate immunity (Bottazzi et al., 2009; Deban et al., 2011; Inforzato et al., 2011). Long pentraxin 3 (PTX3) is the prototype of this family and human PTX3 (HuPTX3) comprise a C-terminal pentraxin-like domain of 203 amino acids (aa) and 178 aa N-terminal domain (Bottazzi et al., 1997; Inforzato et al., 2006).

A variety of cell types express PTX3 upon exposure to inflammatory signals, such as cytokines (*e.g.* IL-1β, TNF-α), toll-like receptor (TLR) agonists, microbial moieties (*e.g.* LPS) or microorganisms (Bottazzi et al., 2009; Cieslik and Hrycek, 2012; Deban et al., 2011; Inforzato et al., 2011). Myeloid dendritic cells (DCs) are the major source of PTX3, although this molecule is also expressed by a number of other cell types (Bottazzi et al., 2010; Cieslik and Hrycek, 2012; Deban et al., 2011; Inforzato et al., 2011). Neutrophils are a reservoir of ready-to-use PTX3, released in minutes, whereas DCs and macrophages produce this molecule *de*

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novo in response to inflammatory signals (Bottazzi et al., 2009; Jaillon et al., 2007). The data indicate that PTX3 is involved in a variety of defence mechanisms, including resistance against some viral infections. Indeed, human and murine PTX3 bind influenza virus (IV) through interaction between viral haemagglutinin (HA) glycoprotein and the sialic acid residue present on PTX3. PTX3 inhibits virus-induced haemagglutination and viral neuraminidase (NA) activity and it neutralizes viral infectivity *in vitro* (Reading et al., 2008). This *in vitro* activity has been also observed *in vivo* in terms of lower mortality and lesser viral load when using an influenza model in mice (Reading et al., 2008). A recent study has shown that both seasonal H1N1 and pandemic H1N1 IVs were resistant to PTX3 (Job et al., 2010). In addition to the specificity of HA, viral NA plays a critical role in determining PTX3 sensitivity, as the sialic acid moiety on PTX3 must resist hydrolysis by NA for neutralization to occur. In spite of the fact that PTX3 plays an important role in host defence, its over-expression can contribute to airway injury (He et al., 2007; Ramery et al., 2010).

The aim of this study was to characterize for the first time *in vitro* and *in vivo* SwPTX3 expression upon infection using human cross-reactive antibodies. This preliminary study will pave the way to understanding SwPTX3 function in pig innate immunity.

2. Materials and methods

2.1. PTX3 and anti-PTX3 antibodies

Recombinant human and murine PTX3 were purified under endotoxin-free conditions by immunoaffinity from the supernatants of stably transfected CHO cells, as previously described (Bottazzi et al., 1997).

Polyclonal rabbit antiserum was obtained by immunization with purified recombinant human PTX3. Specific IgG anti human PTX3 were subsequently purified by immunoaffinity on Cyanogen Bromide (CNBr)-activated Sepharose column conjugated with recombinant human PTX3 (CNBr-activated Sepharose 4 fast flow GE Healthcare, Milan, Italy) (Peri et al., 2000). Rat monoclonal antibodies MNB4 and 16B5 were raised against human PTX3 and shown to recognize respectively epitope (87–99) in the N-terminal domain and epitope (306–312) in the C-terminal domain (Camozzi et al., 2006). Biotinylated antibodies were obtained following incubation with N-hydroxysuccinimidobiotin (Biotin-NHS, Biospa, Milan, Italy). Briefly, purified antibodies (1 mg/ml) in 0.1 M carbonate buffer (pH 8.5) were incubated 4 h at room temperature with Biotin-NHS (1 mg/ml in dimethyl sulfoxide) at the ratio 0.12:1 (v/v). After incubation antibodies were extensively dialyzed against PBS (Bottazzi et al., 1997).

2.2. In silico study

PTX3 sequences from human [GenBank accession number: NP_002843.2], murine [GenBank accession number: NP_033013.3] and swine [GenBank accession number: NP_001231712.1] were retrieved from the National Centre for Biotechnology Information (NCBI) database. Sequences were subjected to blast analysis [http://www.ncbi.nlm.nih.gov/BLAST] and to alignment with Clustal W. (Higgins et al., 1996). Amino acids recognized by MNB4 and 16B5 antibodies in the human PTX3 sequence were compared to the swine PTX3 sequence. NetNglyc server was used to predict N-glycosilation sites [http://www.cbs.dtu.dk/services/NetNGlyc/].

2.3. Swine bone marrow derived dendritic cell generation

Bone marrow hematopoietic cells were obtained from femurs of healthy Large white X Landrace pigs of eight weeks of age. Swine bone marrow dendritic cells (SwBMDCs) were generated in an eight days protocol as previously described by (Crisci et al., 2012; Mussa et al., 2011). Eight-day culture of immature SwBMDCs were used for infection or stimulation.

2.4. Viruses and stimuli

The novel human virus of swine origin, A/Catalonia/63/2009 (H1N1) IV (GenBank accession numbers GQ464405-GQ464411 and GQ168897) was isolated in 2009 from a patient at the Hospital Clinic, Barcelona, Spain, and was propagated in the allantoic cavities of 11 day-old embryonated chicken eggs originating from a commercial specific-pathogen-free (SPF) flock (GDdeventer). Swine influenza virus (SwIV) H3N2 (A/Swine/Spain/SF32071/2007) and H1N2 (A/Swine/Spain/SF12091/2007) were isolated from farms in Catalonia. All influenza viruses were grown and titrated in MDCK cells, and virus titre was calculated by the Reed and Muench method (Reed and Muench, 1938).

For *in vitro* studies, SwBMDCs were infected with 1 multiplicity of infection (moi = 1) with H3N2 SwIV (A/Swine/Spain/SF32071/2007) as previously described (Mussa et al., 2011). In addition, SwBMDCs were stimulated with 10 μg/ml of LPS (Sigma–Aldrich) or 50 μg/ml of virus-like particles belonging to rabbit haemorrhagic disease calicivirus (RHDV-VLPs) (Crisci et al., 2012) for 8 h or overnight (ON). Uninfected and unstimulated SwBMDCs were used as negative control.

2.5. Western blot analysis

SwBMDCs were infected overnight with IV, stimulated with LPS or with RHDV-VLPs. After incubation, cells were washed with 1× PBS and lysed with lysis buffer containing 50 mM Tris-HCL, pH 8, 150 mM NaCl, 2 mM EDTA, 10% Triton X-100 and a protease inhibitor cocktail (Sigma–Aldrich) at 4 °C. After centrifugation at 12,000 × *g* for 15 min at 4 °C, supernatant (lysate) was collected.

Each lysate was generated from 3×10^6 total cells/sample with an average of total protein concentration around 1.7 mg/ml. Fifthteen microlitres of cell lysate (total volume 0.5 ml) were subjected to denaturing SDS-PAGE (NuPAGE® Novex 4–12% Bis-Tris gel; Invitrogen Corporation) followed by Western Blot analysis. Gels were electroblotted to a Hybond ECL™ nitrocellulose membrane (GE Healthcare) followed by blocking in 5% skim milk. After incubation of membranes with primary antibody MNBA or 16B5 (1:10,000 dilution) at room