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Short communication

Enhanced expression of TGFβ protein in lymphoid organs and lung, but not in serum, of pigs infected with a European field isolate of porcine reproductive and respiratory syndrome virus

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ABSTRACT

Transforming growth factor β (TGFβ) is an immunomodulatory cytokine which is able to modulate the host immune response eliciting an inefficient response against pathogens. In this sense, the role of this cytokine in porcine reproductive and respiratory syndrome (PRRS) has been poorly studied and the reported results are contradictory. Thus, in the present study, the expression of TGFβ was analysed both at tissue (lymphoid organs and lung) and serum level to study its correlation with the expression of PRRS virus (PRRSV). To carry out this study, 32 pigs were inoculated with the European PRRSV field isolate 2982 and sequentially killed from 0 dpi to the end of the study (24 dpi). Blood and tissue samples were collected to determine the expression of PRRSV and TGFβ. PRRSV was detected in inoculated animals from 3 dpi until the end of the study, however TGFβ was not detected in sera from inoculated animals. Contrary, an increase of TGFβ antigen was observed both in the lymphoid organs and in the lung of PRRSV-inoculated pigs when compared with control group. Since TGFβ play a role as an immunomodulatory cytokine of the immune response and also in the differentiation of regulatory T cells (Tregs), the upregulation of the TGFβ at tissue level may play a role in the impairment of the host immune response observed during PRRS, being observed a significant correlation between PRRSV and TGFβ expression at lung level.

1. Introduction

Transforming growth factor β (TGFβ) together with interleukin-10 (IL-10) are considered as immunomodulatory cytokines which are able to downregulate the host immune response (Letterio and Roberts, 1998). TGFβ1, one of the three isoforms of TGFβ (Javelaud and Mauviel, 2004), is able to inhibit macrophage activation by means of two mechanisms: (1) the inhibition of the synthesis of IFNγ, which activates macrophages and acts as an antiviral responder (Letterio and Roberts, 1998); and (2) the promotion of the production of IL-10 (Maeda et al., 1995).

Porcine Reproductive and Respiratory Syndrome (PRRS) is a worldwide spread pig disease caused by an arterivirus (Fauquet et al., 2005), known as PRRS virus (PRRSV), which induces an impairment of the host immune response favouring a prolonged viraemia and viral replication (Darwich et al., 2010). Few reports have been focused on the expression of TGFβ during PRRSV infection and the scarce results are controversial. Whereas no enhancement

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in mRNA or protein levels of TGFβ has been reported after infection or vaccination with European or type I PRRSV genotypes (Díaz et al., 2006; Silva-Campa et al., 2010), an increased mRNA and protein expression of TGFβ has been observed in infections with North American or type II genotypes (Silva-Campa et al., 2009; Renukaradhya et al., 2010).

Recently, our group has studied the serum and tissue expression of PRRSV and proinflammatory cytokines in PRRSV-infected pigs, showing a paracrine synthesis of these cytokines associated with a poor serum but a marked tissue expression (Gómez-Laguna et al., 2010a, 2010b; Barranco et al., 2011b). In the present study, samples from those previous experiments were used to analyse the expression of TGFβ both at serum and tissue level in an infection with a European isolate of PRRSV, in order to elucidate its potential role in the immunopathogenesis of PRRS.

2. Materials and methods

2.1. Virus, animals and experimental design

Twenty eight pathogen free, 5-week-old pigs from a PRRSV seronegative farm were randomly assigned to groups of four and inoculated by the intramuscular route with 1 ml of the third passage of PRRSV field isolate 2982 (with an ORF-5 homology of 93% with Lelystad virus; GenBank accession no. EF429108) at $10^{3.0}$ $TCID_{50}$. The inoculated animals were killed at 3, 7, 10, 14, 17, 21 and 24 days post-inoculation (dpi). Another group of four pigs were sham-inoculated controls, which were injected intramuscularly with 1 ml of sterile RPMI 1640 medium and killed at the end of the study (24 dpi). All animals were sedated with tiletamine-zolazepam (Zoletil™; Virbac; Barcelona, Spain) followed by a lethal dose of 5% sodium thiopental (Thiovet™; Vet Limited; Leyland, Lancashire, England). The experiment was carried out according to the guidelines of the European Union (Directive 86/609/EEC) and was approved by the local ethical committee of Centro de Investigación en Sanidad Animal (CISA-INIA; Valdeolmos, Madrid, Spain).

2.2. Clinical signs, viraemia and serum detection of TGFβ

The pigs were monitored daily for clinical signs, i.e. rectal temperature and a clinical respiratory score, as described previously (Gómez-Laguna et al., 2010a).

Blood samples were taken in EDTA-free tubes from eight animals at the different time-points (but the four remaining animals at 24 dpi), and were allowed to clot and centrifuged to obtain serum samples. Virus titration was carried out using an immunoperoxidase monolayer assay (IPMA) as previously reported (Wensvoort et al., 1991).

Serum samples were analysed for TGFβ expression by means of a commercial ELISA kit, following manufacturer's instructions (Swine TGFβ ELISA kit, Biosource). The ELISA kit was carried out using a non-species-specific monoclonal antibody and its sensitivity threshold was 15.6 pg/ml. All samples were analysed in duplicate. TGFβ concentration was calculated by using the linear-regression formula from optical densities of the cytokine standards provided by the manufacturer.

2.3. Histopathology and immunohistochemistry

Samples from the medial lobe of the right lung, mediastinal lymph node and tonsil were fixed in 10% neutral buffered formalin and in Bouin's solution, processed routinely and embedded in paraffin-wax. Four-μm sections of formalin-fixed tissue were stained with haematoxylin and eosin (HE) for histopathology examination.

The avidin-biotin-peroxidase complex technique (ABC) was used in Bouin-fixed samples for the immunohistochemical detection of PRRSV, and TGFβ proteins as described previously (Muñoz et al., 2009; Gómez-Laguna et al., 2010b). Primary antibodies monoclonal anti-PRRSV, clone SDOW-17/SR-30 (Rural Technologies Inc.), diluted 1 in 1000; and polyclonal chicken anti-recombinant human TGF-β1 (R&D Systems, Minneapolis, MN), diluted 1 in 100 were incubated overnight at 4 °C in a humid chamber. In each case, the corresponding biotinylated secondary antibody was incubated for 30 min at room temperature. An avidin-peroxidase complex (Vector Laboratories; Burlingame, CA, USA) was applied for 1 h at room temperature. Labelling was "visualized" by application of the NovaRED™ substrate kit (Vector Laboratories; Burlingame, CA, USA). For negative controls, the primary antibody was replaced by blocking solution, normal serum and isotype-matched reagents of irrelevant specificity.

2.4. Cell counting

The number of labelled cells were counted in 50 non-overlapping and consecutively selected high magnification fields of 0.20 mm^2 (pulmonary parenchyma, paracortex and medulla of mediastinal lymph nodes, and lymphoreticular areas of tonsils) or 25 non overlapping consecutive selected structures (lymphoid follicles of mediastinal lymph nodes and tonsils) for each animal. Results are expressed as the number of cells per mm^2. Immunolabelled cells were identified and counted morphologically as macrophages, lymphocytes, neutrophils or dendritic cells.

2.5. Statistical analysis

All the values are expressed as the mean ± SD. Since control animals were bled at 0, 7, 14, 21 and 24 dpi, blood values of inoculated animals at 3, 10 and 17 dpi were analysed with the mean value of the control animals at the prior- and post-time points. The values of all the studied parameters were evaluated for approximate normality of distribution by using Kolmogorov–Smirnov test. Differences between the means of control and inoculated animals were assessed by the Kruskal–Wallis test followed by the Mann–Whitney-*U* non-parametric test (GraphPad Instat 3.05; San Diego, CA, USA). Correlation between the expression of PRRSV and TGFβ antigens was assessed by the Spearman test (GraphPad Instat 3.05). $P < 0.05$ was considered to represent a statistically significant difference.