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Research paper

Lawsonia intracellularis infection of intestinal crypt cells is associated with specific depletion of secreted MUC2 in goblet cells

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ABSTRACT

The expression patterns of secreted (MUC2 and MUC5AC) and membrane-tethered (MUC1, MUC4, MUC12 and MUC13) mucins were monitored in healthy pigs and pigs challenged orally with *Lawsonia intracellularis*. These results showed that the regulation of mucin gene expression is distinctive along the GI tract of the healthy pig, and may reflect an association between the function of the mucin subtypes and different physiological demands at various sites. We identified a specific depletion of secreted MUC2 from goblet cells in infected pigs that correlated with the increased level of intracellular bacteria in crypt cells. We concluded that *L. intracellularis* may influence MUC2 production, thereby altering the mucus barrier and enabling cellular invasion.

1. Introduction

Lawsonia intracellularis (*LI*) is a Gram-negative intracellular bacterial pathogen; the aetiological agent that causes proliferative enteropathy (PE) (Jacobson et al., 2010). *LI* causes both subclinical infection and clinically apparent disease. Both forms affect the well-being of animals, clinically manifesting as poor feed conversion, anorexia, diarrhea and occasionally fatalities due to severe hemorrhage of the intestinal tract. This infection therefore affects health, welfare and productivity of pigs and is also increasingly reported in a wide range of animals, particularly in horses. *LI* infects the intestinal crypts and invades the immature enterocytes, causing intestinal hyperplasia by stimulating continuous cell division (Smith and Lawson, 2001) and alteration of mucosal cell integrity (Smith et al., 2014; Vannucci et al., 2013; Vannucci and Gebhart, 2014).

The mucosal epithelial tissues have highly specialized functions throughout the gastrointestinal (GI) tract to allow food absorption and processing for excretion. They also serve as a continuous barrier both to the commensal microorganisms and to potential viral, bacterial and eukaryotic pathogens (McGuckin et al., 2011). These epithelial cells constitutively secrete a highly hydrated structural mucus barrier that coats the surfaces of the cells lining the GI tract. This layer of complex fluid consists of a vast repertoire of defense compounds including mucin glycoproteins, antibodies, defensins, protegrins, collectins, cathlecidins, lysozymes, histatins, and nitric oxide. Regulated by underlying innate immune cell defense, the mucus barrier can also respond dynamically to pathogen insults, by altering its production rate, constituents and biophysical properties (Atuma et al., 2001). Mucins are the main component of the viscous layer and are synthesized and secreted by goblet cells which are scattered within the epithelial lining (Kim and Khan, 2013). The mucin gene family comprises 20 different mucin genes ranging from MUC1 to MUC20. They are classified as secreted and membrane bound forms (Bansil and Turner, 2006). Mucins are a family of highly glycosylated macromolecules that are multifunctional and provide the epithelial cells with protection against physical or chemical injures and mechanical stress, lubricating the intestinal tract to ease the passage of food content and preventing mucosal dehydration Bansil and Turner, 2006. Their primary function, however, is to serve as a first line of defence against pathogens such as *Yersinia enterocolitica* and *Shigella flexneri* (Kim and Khan, 2013; Mantle and Husar, 1994; Nutten et al., 2002). Mucins have been shown to have direct antimicrobial activity, and the ability to opsonize microbes to aid clearance. However, growing evidence suggests that intestinal bacteria have developed specific pathogenic factors and/or ways of interfering with mucin

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production in order to enable pathogens to cross the physical mucus barrier. Such a phenomenon has been observed during *Helicobacter pylori* invasion of the human stomach through its interaction with MUC5AC, enabling the bacteria to colonize and reside in the gastric mucus layer (Van de Bovenkamp et al., 2003). This is followed by alteration of MUC1 and MUC6 production to weaken the mucus gel layer, allowing the bacteria to penetrate through to the epithelial cells below (Byrd et al., 2000; Morgenstern et al., 2001; Van de Bovenkamp et al., 2003). Increased expression of MUC5AC in pig colon has also been observed during salmonellosis (Kim et al., 2009). Alteration of mucin expression has been shown in several diseases such as cystic fibrosis, chronic gastric inflammation caused by *H. pylori* and certain cancers (Llinares et al., 2004; Roger et al., 2000; Rose and Voynow, 2006). Such observations have highlighted the importance of mucins in relation to diseases and infections, and led us to examine in greater detail the changes in the regulation of mucin gene expression in the pig GI tract, following infection with *LI*. Recent work conducted by Smith et al. revealed that the gene encoding the glycoprotein MUC2 was down-regulated at the time of peak *LI* infection burden, suggesting that this pathogen may have a profound impact on mucin production and echoing previous research (Driemeier et al., 2002; Smith et al., 2014).

The objective of this work was to measure the pattern of MUC1, MUC2, MUC4, MUC5AC, MUC12 and MUC13 expression in the GI tract of healthy pigs and in the ileum of *LI* infected pigs. We found that the accumulation of MUC transcripts were highly regulated during *LI* infection and that the specific depletion of secreted MUC2 from goblet cells correlated with the increased level of intracellular bacteria in crypt epithelial cells.

2. Materials and methods

2.1. Samples

Tongue, stomach, duodenum, ileum, caecum, colon and rectum tissues were isolated from healthy Duroc pigs ($n = 2–4$) aged between 5 and 7 months with male and female equally represented. Infected ileum samples originated from a previous challenge study by MacIntyre et al. (2003), in which pigs were randomly selected from a minimal disease herd and fecal samples were culture negative for *Brachyspira hyodysenteriae*, *B. pilosicoli*, *Yersinia* spp, *Salmonella* spp and *LI*. Pigs were challenged with a pure culture of *LI* (isolate LR189/5/83) and euthanized at 3, 7, 14, 21, 28, 35 or 42 days post challenge (dpc). All pigs were then subjected to full necropsy. Infection was confirmed immunohistochemically using a monoclonal antibody VPM53 against *LI* (MacIntyre et al., 2003). For data normalization, negative control ileum samples were obtained from a separate group of three uninfected age-matched pigs.

2.2. RNA extraction

Total RNA was extracted from the ileum of infected pigs; these samples had been used previously for a microarray analysis (Smith et al., 2014). Briefly, total RNA was extracted from the ileum of infected pigs with Trizol (Invitrogen) using standard methods (Ait-Ali et al., 2007). The RNA was further purified using the Qiagen RNeasy mini-kit following the manufacturer's protocol (Qiagen). Using a Nanodrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and Agilent 2100 bioanalyser (Agilent Technologies) the quantity and quality of RNAs were assessed.

2.3. Quantification of L. intracellularis-specific genomic DNA

Genomic DNA of ileal samples for the measurement of *L. intracellularis*-specific genomic DNA originated from previous experiment (Smith et al., 2014). Quantitative measurement was performed as described previously and was expressed as number of 16S rRNA copies per ng of DNA (Smith et al., 2014). Only DNA from pigs at 3, 14 and 28 dpc were selected because they showed statistically significant variations ($P < 0.0003$) from the peak of infection at 14 dpc. Primers used are described in supplementary material 1.

2.4. Quantitative RT-PCR validations

Expression of MUC transcript levels in healthy and infected tissues was measured using quantitative RT-PCR (qRT-PCR). To reduce the likelihood of amplifying any contaminating genomic DNA that may be present in RNA preparations, MUC specific primer sequences (supplementary material 1) were positioned on exons flanking an intron, and qRT-PCR was run at extension times that favored the amplification of shorter cDNA sequences. RNA samples were analyzed with Brilliant III Ultra-Fast SYBR Green qRT-PCR Master Mix kit (Agilent Technologies) according to manufacturers' instructions. Quantitative RT-PCR was performed with a Stratagene MX3000P (Stratagene) using the following profile: 48 °C 10 min, 95 °C 3 min; 40 cycles of 95 °C 15 s, 60 °C 20 s, and 95 °C 60 s, 60 °C 30 s, 95 °C 15 s, 25 °C 30 s. Samples were tested in triplicate.

A standard curve was used to quantify mucin transcript in the total RNA samples. Briefly, standard curves were constructed using gBlocks® Gene Fragments (Integrated DNA Technologies) of mucin gene sequences (Gene fragment sequences are listed in supplementary material 2), fused to a T7 promoter. The gBlocks® Gene Fragments were transcribed into cDNA through in vitro transcription, using mMESSAGE mMACHINE T7 transcription kit (Life Technologies, Paisley) according to the manufacturer's instructions. Agarose gel electrophoresis was used to verify transcribed products, followed by lithium chloride precipitation to purify the transcribed gBlocks Gene fragments mixture. Quality and quantity of the DNA was assessed using a Nanodrop spectrophotometer. Serial dilutions of the transcribed gene fragment were run on each plate of samples and analyzed by qRT-PCR. The log of the serial diluted gBlock gene fragment quantities was plotted against Cycle Threshold values to generate a standard curve, which was used to estimate the quantities of MUC transcript copy number in each sample.

2.5. Periodic acid Schiff staining

Sections of paraffin-embedded ileum from pigs euthanized at 3, 14, 28 dpc, and uninfected control pigs were processed, deparaffinized and hydrated according to standard protocols (Smith et al., 2014). Sections were treated for 5 min in 0.5% periodic acid and rinsed in distilled water. Sections were treated with Schiff's reagent at room temperature for 30 min and extensively rinsed in running tap water for 5 min. After mounting, sections were observed using a standard light microscope.

2.6. Immunohistochemistry

Sections of paraffin-embedded ileum from pigs euthanized at 3, 14, 28 dpc, and uninfected control pigs were processed, deparaffinised and hydrated according to standard protocols (Smith et al., 2014). Due to the fact that the detection of *LI* and MUC antigens required different method of antigen retrieval (see below) a dual staining strategy was not an option. Therefore we opted for single antigen detection on contiguous sections. Sections stained to detect *LI* were incubated for 10 min in proteinase K (Dako UK Ltd., Ely, UK) to allow antigen retrieval. After 30 min incubation in blocking agent (5% BSA/2% goat serum), the sections were incubated with mouse anti-*LI* VPM53 (MacIntyre et al., 2003) diluted 1:400 in blocking solution, at 4 °C overnight. After two washes in PBS, the slides were incubated for 30 min at room temperature with a FITC-conjugated