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Microarray analysis of differential expression of cell cycle and cell differentiation genes in cells infected with *Lawsonia intracellularis*

Yu-Sik Oh [a], Joong-Bok Lee [a], Steven McOrist [b,*]

[a] Laboratory of Veterinary Immunology and Microbiology, College of Veterinary Medicine, Konkuk University, Seoul, South Korea
[b] School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington, Leics, United Kingdom

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ABSTRACT

Infection of intestinal crypt epithelial cells by the obligate intracellular bacterium *Lawsonia intracellularis* is directly linked to marked proliferation of the infected enterocytes within 3–5 days post-infection. The virulence factor for this unique host cell-proliferative response is not known, but is considered to involve altered crypt cell cycle or differentiation events. McCoy mouse fibroblast cells were infected with *L. intracellularis*, and then harvested for expressed mRNA at daily time points, with matching non-infected control cell cultures.

Mouse DNA microarray (>44,000 transcript targets) analysis of cDNA derived from matching mRNA samples showed over 40 identifiable genes with at least 4-fold changes between days 0 and 3 after infection with *L. intracellularis*. These included altered transcription of typical host cell 'alarm' response genes, such as interferon-related response genes *Isgf3g* and *Igtp*, known to be associated with invading microbial agents. Altered transcription of several genes in these cells known to be active in regulation of the cell cycle or cell differentiation genes, including *usp18*, *Hr*, *Elavl2* and *Slfn2*, were also detected. The altered transcription of several of these genes via RT-PCR analysis was confirmed. The microarray-detected altered transcription of cell cycle and cell differentiation genes is of possible interest for links to *Lawsonia*-related disturbances in epithelial cell differentiation within the intestinal crypt, but this would need to be confirmed in intestinal epithelial cell studies.

Introduction

The pathogen *Lawsonia intracellularis* is a Gram-negative, obligate intracellular bacterium that is normally non-membrane-bound and resides in the cytoplasm of immature epithelial cells in the lower intestine of weaned pigs and other hosts (McOrist et al., 1995a). Uniquely amongst known proteobacteria, this intracellular bacterial infection causes marked monotypic proliferation of the infected epithelial cells, unrelated to an inflammatory response (McOrist et al., 1989, 1993). The characteristic pathological feature is therefore the hyperplastic proliferation of immature epithelial cells within the crypts of the ileum and/or colon, leading to thickened, branched crypts and gross mucosal thickening, sometimes described as an adenomatous reaction (Frisk and Wagner, 1977; McOrist et al., 1993). However, the mucosal hyperplasia recedes to normal epithelium if the *L. intracellularis* infection of the cells is removed (McOrist et al., 1996).

The clinical signs in affected post-weaned animals can include diarrhoea, weight loss and melaena (Frisk and Wagner, 1977; Guedes and Gebhart, 2003) in the specific disease known as proliferative enteropathy or ileitis. It is a common enteric disease of post-weaned pigs worldwide and an identical infection and disease has been found in certain other animals, particularly horses, rabbits and hamsters, but not in other animals such as chickens or humans (McOrist et al., 1995a).

L. intracellularis can be co-cultured in static monolayers of transformed intestinal epithelial cells derived from rats or pigs (Lawson et al., 1993; McOrist et al., 1995b). It was found that co-cultures using a murine fibroblast cell line were easier to expand and manipulate (Guedes and Gebhart, 2003; Yeh et al., 2006). However, these current in vitro cell co-culture models of *L. intracellularis* infections do not display any striking cytopathic effects; in particular they lack recognisable cell proliferation (Lawson et al., 1995; McOrist et al., 1995b). Also, static monolayers of transformed or primary intestinal epithelial cell cultures, whilst capable of supporting moderate *L. intracellularis* growth, are not yet capable of providing sufficient DNA quantities for bulky molecular analysis methods. Therefore when considering the co-culture of *L. intracellularis* for molecular studies requiring large amounts of DNA such as gene sequencing, murine fibroblast co-cultures are favoured.[1]

* Corresponding author. Tel.: +44 115 951 6430; fax: +44 115 951 6412.
E-mail address: steven.mcorist@nottingham.ac.uk (S. McOrist).

[1] See: http://www.genome.jp/kegg-bin.

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The virulence mechanism whereby intracellular *L. intracellularis* causes a monotype of infected mammalian intestinal crypt epithelial cells to form hyperplastic or adenomatous features is not known. A review of possible mechanisms of *L. intracellularis* virulence (McOrist et al., 1996) suggested one or more of (1) a mitogenic disturbance of crypt epithelial cell cycle processes; (2) a disturbance of factors involving the differentiation of the normally immature and proliferative crypt cells into mature villous cells at the neck region of the crypt-villus unit; (3) a disturbance of relevant growth factor receptors and/or chemokines that control crypt-villus unit height and repair, and (4) a disturbance of apoptotic cell death in the crypt.

The direct and recoverable nature and the location of the linked proliferation and intracellular *L. intracellularis* infection are suggested to favour factors (1) and/or (2) above (Lawson et al., 1995; McOrist et al., 2006), indicating a direct *Lawsonia*-induced disturbance of the cell cycle and/or differentiation events within the infected crypt epithelial cells. The sequencing of the entire 1.7 Mb genome of *L. intracellularis*[1] has so far failed to suggest particular virulence genes or products in this regard. The present study aimed to explore the differential expression genetic events during infection of cells with *L. intracellularis*, with an emphasis on cell cycle genes.

Previous studies of co-cultures of several other obligate intracellular bacteria (not associated with monotypic host cell proliferation) have indicated that these bacteria induce a typical host cell 'alarm response'. This occurs when cells are attacked by most infectious agents and consists of the induction of numerous inflammatory-related, interferon-related, immune response and other genes (Hess et al., 2003; Jenner and Young, 2005; Granberg et al., 2006). We aimed to generate a preliminary list of genes clearly active (>2-fold change) in the active intracellular stages of *L. intracellularis* infection of mouse cells by screening infected cells by a microarray technique.

Materials and methods

Co-culture of L. intracellularis isolate in McCoy cells

Pure co-cultures of the *L. intracellularis* isolate PHE/KK421 were initially derived from the intestinal mucosal lesions of a pig confirmed as affected with proliferative enteropathy, using bacterial purification and co-culture techniques described previously (Lawson et al., 1993; Yeh et al., 2006). The isolate is deposited in the Korean Collection for Type Cultures as KCTC 10686BP.

Purified bacterial suspensions were routinely seeded at 5×10^5 mL^{-1} onto fresh 1 day-old cultures of murine fibroblast-like McCoy cell (ATCC CRL 1696) grown in DMEM (Dulbecco's modification of Eagle's medium) with 1% L-glutamine and 5% FBS (foetal bovine serum) without antibiotics, at 37 °C in microaerobic conditions (8.0% oxygen, 8.8% carbon dioxide and 83.2% nitrogen). Co-cultures used for each experiment were checked for purity by routine aerobic and anaerobic bacterial culture. Confirmation of full viability of cell infection and counting of *L. intracellularis* within suspensions and co-cultures was performed by *L. intracellularis*-specific PCR and by immunostaining of sentinel portions with *L. intracellularis*-specific monoclonal antibody. Illustrations of typical immunostained 5-days-old cell cultures in our laboratory, with or without infections of 1.5×10^7 *L. intracellularis*, identical to those used in this study have been published previously (Yeh et al., 2006). Cohort co-cultures had been previously established as capable of pathogenic infections of laboratory animals (Go et al., 2005). Uninfected McCoy cells were maintained in adjacent cultures as control sources of mRNA and harvested at the appropriate number of days of non-infected growth for comparison with infected co-cultures.

RNA extraction

Infected and non-infected McCoy cell cultures were usually harvested at 1-day intervals for 3 days after initial co-culture infection, placed in separate RNase-free tubes and immediately pelleted and lysed with denaturing guanidine iso-thiocyanate buffer. After addition of ethanol, total RNA from each pellet were extracted using the RNeasy Mini Kit column and washing steps according to the manufacturer's instructions (Qiagen). Each RNA sample was analysed for purity and concentration by laser-induced fluorescence (Agilent 2100 bioanalyzer).

Mouse DNA microarray

We used mouse genome survey microarray chips containing 32,996 60-mer DNA gene probes, representing 44,498 transcript targets or gene markers (Applied Biosystems). The mouse genome has approximately 33,000 genes and 55,000 gene markers, so the chips were considered nearly complete. Total RNA extracted from infected and non-infected co-cultures was converted to cDNA and reacted with separate chips according to the manufacturer's instructions, in two biologically-separate rounds. Briefly, digoxigenin-11-UTP labelled cDNA was generated from 1 μg of each total RNA and linearly amplified using the chemiluminescence RT-IVT labelling reagents supplied. Each microarray chip was pre-hybridised at 55 °C for 1 h in the hybridisation and blocking buffers supplied. Sixteen micrograms of each labelled cDNA target was fragmented at 60 °C for 30 min, in the reagents designed to generate 100–400 base fragments, and then mixed with an internal 24 mer labelled control target.

Each specimen sample was hybridised to the prepared chip at 55 °C for 16 h, and then processed with wash and chemiluminescence rinse buffers, and with anti-digoxigenin-alkaline phosphatase, enhancing solution and substrate. Each chip's chemiluminescence gene expression image was collected on the Applied Biosystems 1700 analyser at both 5 and 25 s exposures. Images were auto-gridded and signals quantified, corrected for background and spot and spatially normalised using the Applied Biosystems Expression System software, such that computer-generated dot-charts could be aligned and analysed.

Mouse DNA microarray analysis

Raw image data files from each chip were analysed with the Avadis software (Strand Genomics). The assay signal of the usable sets of 99.5% of genes was log transformed and filtered for a signal to noise ratio of >3 leaving 57% of genes (n = 18,800) remaining for analysis of fold change, when *L. intracellularis*-infected were compared to the control non-infected co-cultures. One-sample signed rank tests were used to test that the medians of log fold change populations were different to hypothesis values defined as maximum or minimum of 2-fold change expected on replica arrays. Only genes with P-values <0.05 were considered candidates for differential expression. To further reduce false differential expression, the candidate genes were filtered by assay signal-dependent log fold change threshold measurements across 10 sets of inter-quantile ranges for each chip. Only genes falling outside a corresponding inter-quantile range were included in final lists of differential expression. Gene identity was confirmed by analysis of GenBank (incorporating Celera mouse genome sequences), in other words sequence data linked to the published mouse genome databases.[2]

Confirmation of differential expression of genes

The expression of selected genes was further analysed for *L. intracellularis*-infected McCoy cells by reverse transcription (RT)-PCR of extracted mRNA. Two negative controls, including a –RT control without reverse transcriptase and a minus template RNA, were used. Total cellular mRNA (2 μg) was used for the first-strand cDNA probe synthesis, extracted as described above at the three points after co-cultured cell cultures were infected, up to 3 days post-infection and compared to two endogenous controls for input RNA (β-actin and P-selectin) to normalise for input RNA.

Customised PCR probes for each of the selected study genes (including putative up- or down-regulated genes), each containing 5′ fluorescein phosphoramidite reporter dye-labelled gene-specific sequences, and 3′ minor groove binder moiety as quencher, or left unlabelled, were designed and supplied by a commercial manufacturer (Assays-by-Design, Applied Biosystems). Each mRNA test and control sample was reacted in triplicate with the customised probes (0.25 μM) and unlabelled PCR primers (0.9 μM), in a total volume of 25 μL in 40 sequential PCR cycles in a commercial RT-PCR system (Cepheid Smart Cycler). Each cycle comprised 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min and 40 cycles of 95 °C for 15 s and of 60 °C for 60 s. The relative expression levels of control genes and test mRNA were calculated via the comparative C_T value data analysis for each gene using the manufacturer's software. The selected host cell genes and their primers are listed in Table 1.

Results

Mouse DNA microarray

Mouse oligonucleotide microarray hybridisation was performed on three separate occasions with RNA prepared from duplicate biologically-separate *L. intracellularis*-infected and uninfected cells. Genes with expression levels that changed in response to the infection were selected on the basis of repeated differences in the

[2] See: http://www.ncbi.nlm.nih.gov.