Differential expression of groEL-1, incB, pyk-F, tal, hctA and omcB genes during Chlamydia trachomatis developmental cycle

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Abstract

Chlamydia trachomatis infects squamous and columnar epithelia at the mucosal surface. Research on gene expression patterns of C. trachomatis has predominantly focused on non-native host cells, with limited data on growth kinetics and gene expression of chlamydia in keratinocytes. Here, we investigated whether early, mid, and late chlamydial genes observed in HeLa cell line studies were co-ordinately regulated at the transcriptional level even in the keratinized cell line model and whether the expression was stage-specific during the developmental cycle. HaCaT cell lines were infected with chlamydia clinical isolates (US151 and serovar E) and reference strain (L2 434). Expression of groEL-1, incB, pyk-F, tal, hctA, and omcB genes was conducted with comparative real-time PCR and transcriptional events during the chlamydial developmental cycle using transmission electron microscopy. The relative expression level of each gene and fold difference were calculated using the $2^{-\Delta\Delta CT}$ method. The expression of groEL-1 and pyk-F genes was highest at 2 hours post-infection (hpi) in the L2 434 and serovar E. The expression of incB gene increased at 2 hpi in L2 434 and serovar E but peaked at 12 hpi in serovar E. L2 434 and US151 had similar tal expression profiles. Increased expression of hctA and omcB genes were found at 2 and 36 hpi in L2 434. Both clinical isolates and reference strains presented the normal chlamydial replication cycle comprising elementary bodies and reticulate bodies within 36 hpi. We show different gene expression patterns between clinical isolates and reference strain during in vitro infection of keratinocytes, with reference strain-inducing consistent expression of genes. These findings confirm that keratinocytes are appropriate cell lines to interrogate cell differentiation, growth kinetics, and gene expression of C. trachomatis infection. Furthermore, more studies with different clinical isolates and genes are needed to better understand the Chlamydial pathogenesis in keratinocytes.
Introduction

In 2018, more than a million *Chlamydia trachomatis* infections a day were reported to the World Health Organization, making it a major public health concern in both developed and developing countries [1]. *Chlamydia spp.*, commonly infect a more vulnerable single layer of simple columnar epithelium or non-keratinized stratified squamous epithelium found in the endocervix and transformation zone of the female genital tract [2, 3]. The *C. trachomatis* infection of the genital tract is characterized by sexually transmitted diseases such as oculogenital and lymphogranuloma venereum (LGV) [4, 5]. The ability of this pathogenic bacterium to cause disease is related to its unique two-phase developmental cycle, which takes place in the host cell. In the extracellular phase, the organism manifests as the elementary body (EB), an extracellular and metabolically inert form of *C. trachomatis* that targets columnar or squamous cells lining the mucosal epithelium by receptor-mediated endocytosis [6]. Once the EB is endocytosed it differentiates into the metabolically active reticulate body (RB) which replicates within inclusions by binary fission [6–9]. After many rounds of binary fusion, the majority of RBs convert to the metabolically inactive EBs that are released from the host cell to infect neighboring cells [6–8].

Several studies have shown that multiple chlamydial genes are temporally expressed and regulated by specific mechanisms during the developmental cycle [10–12]. *De novo* transcription and translation are required to facilitate and coordinate molecular events occurring during the developmental cycle [6]. *In vitro* analysis suggest that the *C. trachomatis* developmental cycle is regulated at the transcriptional level in three temporal phases, with early classes of genes expressed within 2 hours post endocytosis, mid-cycle genes within 6–24 hours post-infection (hpi) and during RB replication, and late genes within 24–48 hpi when a majority of RBs convert to EBs [10, 11]. For example, *incB* and *groEL* are genes expressed at high levels in the early stages of the chlamydial infection and continue to be expressed in a low level throughout the developmental cycle [11, 13]. This regulated expression of early genes suggests that they play an important function in the initial stages of infection and not necessarily throughout the developmental cycle. Mid-cycle genes [*ompA*, pyruvate kinase (*pyk*) and transaldolase (*tal*)], associated with energy metabolism, are crucial immunogenic determinants for different serovars and subspecies [10, 14–16]. Late genes such as *omcA*, *omcB*, *hctA*, and *hctB* have functions associated with the morphologically dramatic events that occur 24 hpi when RBs convert to EBs. This is confirmed by two abundant cysteine-rich proteins that form part of the outer membrane of EBs and not RBs [17]. In addition, late genes *hctA* and *hctB* encode lysine-rich proteins with a primary sequence similar to the eukaryotic histone Hc1 and Hc2, which mediate the compression of DNA observed when RBs convert into EBs [16, 18–20]. Taken together, this suggests that the temporal expression profile of chlamydial genes demonstrates that genes are transcribed only at a time in the developmental cycle when they are needed.

Although research on pathogenesis and transcriptional expression of genes of *C. trachomatis* is well characterized, it is predominantly conducted in monolayer cultures of epithelial cells such as cervical cancer HeLa cells, which are different from the lower mucosal epithelium infected *in vivo* [10, 21, 22]. There is a growing body of evidence that keratinocytes, a primary target of infection for *C. trachomatis* LGV biovar, can support the growth of *C. trachomatis* in the *in vitro*-generated monolayers of immortalized human keratinocyte (HaCaT) cells [2, 4, 23]. Previously, our group has shown that *C. trachomatis* LGV biovar L2 grew significantly faster than LGV biovar L1 and L3 in HaCaT cells [23]. Nogueira *et al.* (2017) also observed the optimal growth of *C. trachomatis* seeded in the stratified squamous epithelium [2]. Furthermore, another study assessing the effects of *Chlamydia* type III effector TarP on epithelium...
was able to grow *C. trachomatis* in various cell types including HaCaT cells [24]. HaCaT cells are one of the cell lines than can be used to interrogate cell differentiation, growth kinetics, and gene expression of *C. trachomatis* infection.

While keratinocytes are optimal cells for chlamydial growth, none of the temporally regulated genes expressed during the chlamydial developmental cycles were investigated in studies using non-transformed keratinocyte epithelial cells, which are important native host cells to gain insights into the *C. trachomatis* cell biology and pathogenesis during infection of squamous epithelium. In this study, we tested the hypothesis that the three temporal classes of early, mid, and late chlamydial genes observed in HeLa cell line studies are co-ordinately regulated at the transcriptional level even in the keratinized cell model. In addition, we determined whether expression of *groEL-1*, *incB*, *pyk-F*, *tal*, *hctA* and *omcB* genes during *in vitro* infection of the HaCaT cells were stage-specific during the developmental cycle.

**Materials and methods**

**Cell line and *Chlamydia trachomatis* strains**

The HaCaT cell lines (kindly donated in 1995 by Professor N. E. Fusenig of the Cancer Research Centre, Heidelberg, Germany) were used for both propagation of chlamydia and the experimental work. Two *C. trachomatis* LGV and one genital strain were used for the experiments: the L2 reference strain 434 (ATCC VR-902 BTM), one serovar L2 clinical isolate (US151), and one serovar E clinical isolate, respectively. US151 and serovar E were isolated in the male patient presenting with genital ulcer and urethritis at the Prince Cyril Zulu Communicable Diseases Clinic in Durban, South Africa.

**C. trachomatis culture and infectious particle recovery**

*C. trachomatis* strains were propagated in HaCaT cell monolayers. Briefly, HaCaT cells grown in 12-well plates (2x10⁶ cells per well) were infected with *C. trachomatis* in RPMI-1640 supplemented with glucose (5.4 mg/ml), 10% FBS, 10 mM HEPES, 2mM L-glutamine, gentamicin (10 μg/ml) and amphotericin-B (5 μg/ml). Cultures were centrifuged for 1 hour and then incubated for another hour at 37°C, 5% CO₂. Media change was performed after the 1-hour incubation, then cultures containing fresh medium were incubated at 37°C with 5% CO₂ for 48 hours. After 48 hours of growth, infected host-cell monolayers were lysed and chlamydia was harvested in sucrose phosphate glutamate (SPG) buffer. Triplicates of infected HaCaT cell monolayers cultured with tenfold serial dilutions of the inoculum were used to determine the infectious titer. *C. trachomatis* LGV L2 434, L2 US151, and strain E were used to infect the HaCaT monolayers at a multiplicity infection (MOI) of 10 and incubated at 37°C. The number of chlamydial inclusions was enumerated, and the number of inclusion-forming units/ml was calculated. Infection was confirmed using the MicroTrak *C. trachomatis* Culture Confirmation Test kit (Trinity Biotech) and fluorescence microscopy.

**Isolation and amplification of *C. trachomatis* genes**

Infected HaCaT cells lysed in 2 ml/well of guanidine thiocyanate (GTC) solution containing 1.4% β-mercaptoethanol (Sigma, Steinheim, Germany) and 200 μl Trisure (Bioline, London, UK) were used to isolate total *C. trachomatis* RNA harvested at 2, 12, 24, 36 and 48 hpi. Complementary DNA was generated using adjusted RNA (0.2 μg/μl) and the high capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies. *C. trachomatis* genes (*groEL-1*, *incB*, *pyk-F*, *tal*, *omcB* and *hctA*) were amplified by comparative real-time PCR, using primers (Table 1), PCR conditions and ran on ABI Prism 7500 Real-Time PCR System (Applied
Transmission Electron Microscopy (TEM)

*C. trachomatis* infected HaCaT cells grown on 12 mm Nunc™ Thermania™ coverslips (ThermoFisher, USA) were fixed with 2% glutaraldehyde in EMEM (BioWhittaker™, Walkersville, USA), followed by two washing steps with EMEM and one step with 0.1M sodium cacodylate buffer, pH 7.4. The cells were fixed with 1% osmium tetroxide, dehydrated with a graded series of ethanol (50, 70, 90, and 100%) at 24˚C, infiltrated, and embedded in Spurr’s resin (Sigma, Steinheim, Germany) overnight at 60˚C (S1 Table). A beam capsule filled with Spurr resin was used to embed the fixed cells for TEM. Ultrathin sections (50–60 nm) were cut and collected onto 3.05 mm diameter, square uncoated mesh copper TEM grids, then double-stained with uranyl acetate and Reynold’s lead citrate (Sigma, Steinheim, Germany) for 10 minutes each [25]. Sections were viewed using a Jeol 1010 transmission electron microscope (JEOL Ltd) at an accelerating voltage of 100 kV. The TEM was interfaced with a Megaview III Software Imaging Systems camera unit (Soft Imaging System, Münster, Germany). Images were captured digitally, and measurements performed using iT EM analySIS (Soft Imaging System, Münster, Germany) image analyzing software.

Data analysis and validation analysis of standard curves for *C. trachomatis* genes

All experiments were carried out three times in triplicates. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression level of each gene from the real-time quantitative PCR experiments. Briefly, the mean ΔCT values were calculated by subtracting the mean target CT value from the mean 16S rRNA reference CT value using the formula: $\Delta CT = \Delta CT_{test \ sample} - \Delta CT_{calibrator \ sample}$. Furthermore, the standard deviations of the $\Delta\Delta CT$ values were the same as the standard deviation of the ΔCT value. This study did not formally test any hypothesis and therefore could not produce any p-values due to limited data points. However, we adopted a simple analytical approach by incorporating standard deviations of the $\Delta\Delta CT$ values into the fold difference calculation ($\Delta\Delta CT \pm s$) [26] and calculated the relative
difference between target and reference values. All analyses were conducted using Microsoft Excel (Microsoft, USA) and GraphPad Prism 8 (GraphPad Software, USA).

Results

Differential expression of *C. trachomatis* genes at 37˚C post-infection by serovars

Here, we determined whether HaCaT gene expression patterns differed between clinical isolates and reference strain and whether the expressions were time-dependent. Overall, we observed differences in the gene expression patterns between clinical isolates and reference strain (Fig 1 and S2 Table). There were continuous expression of groEL-1, incB, and pyk-F genes while hctA, tal, and omcB genes were expressed at low levels following infection of HaCaT cells with all strains. Early- (groEL-1) and mid-cycle (pyk-F) genes were expressed between 2 and 48 hpi and the expression peaked by more than 90 fold at 2 hpi with L2 434 and
US151 (L2). In addition, groEL-1 and pyk-F genes were moderately expressed in serovar E. The expression of the early-cycle gene incB was high with more than 30 fold at 2 hpi in L2 434 and at 12 hpi in US151 (L2). There was a less than 7 fold expression of tal and 4 fold expression of omcB gene at 2 and 12 hpi in serovar E stimulated cells. Although modest, the expression of late-cycle genes hctA and omcB were observed at 2 hpi and the expression of omcB gene peaked at 36 hpi in L2 434. Low expression levels of tal, hctA and omcB were observed in US151 (L2). Similarly, L2 434 did not induce high levels of tal gene. A similar expression profile (4 fold) of omcB was observed between 2 and 12 hpi in cells infected with serovar E. Some of the genes in response to serovar infection could not be expressed at 2 (strain L2: tal), 12 (strain L2: incB, tal, omcB; serovar E: hctA; US151: tal, omcB), 24 (serovar E: omcB; US151: tal, omcB), 36 (serovar E: incB, pyk-F, tal, hctA, omcB; US151: tal, omcB), 48 hours post-infection (strain L2: tal, hctA, omcB; serovar E: tal; US151: hctA) using qPCR.

Expression of *C. trachomatis* groEL-1, pyk-F and hctA genes in the HeLa cells

We further verified the observations in HaCaT cells using HeLa cells. Fig 2 summarizes the results of RT-PCR analysis in infected HeLa cells for three chlamydial genes (groEL-1, pyk-F, hctA) representing each of the three proposed temporal classes of chlamydial gene expression. The findings of this study were in keeping with the published data [10], with groEL-1, pyk-F and hctA detected between 2 and 48 hpi at 37˚C in both cell lines. HaCaT cells expressed high levels of groEL-1 and pyk-F genes at 2 and 24 hpi compared to HeLa cells. Expression of hctA gene at 24 hpi was more pronounced in HeLa compared to HaCaT cells and continued to 48hpi with L2 434 and US151 (L2). Some of the genes in response to serovar infection could not be expressed at 2 (strain L2: pyk-F, hctA; US151: pyk-F, hctA) and 12 hours (L2: hctA; US151: hctA) post infection using qPCR, which is in keeping with published data.

Fig 2. The expression groEL-1, pyk-F and hctA genes of *C. trachomatis* reference strain L2 434 and clinical isolates US151 (L2) and serovar E at 2, 12, 24, 36 and 48 hpi in HaCaT and HeLa cells at 37˚C. Bar charts representing the mean fold difference (2^(-ΔΔCT)) of genes and the results are based on experiments carried out three times in triplicates.

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The developmental cycle of *C. trachomatis* in human keratinocytes

To place transcriptional events of *groEL-1, incB, pyk-F, hctA, tal* and *omcB* genes into the context of the *C. trachomatis* LGV and genital biovars developmental cycle, we conducted an ultrastructural analysis of the chlamydial developmental cycle in HaCaT cells using TEM. Both LGV (L2 434 and US151) and the genital biovar (serovar E) presented the normal chlamydial replication cycle comprising EB and RB. Fig 3 shows the ultrastructural events observed during *C. trachomatis* LGV and genital biovars developmental cycle at 37°C. Fig 3 shows the ultrastructural events of strain L2 434, US151 (L2), and serovar E. Within Fig 3, panel A1, B1, and C1 show uninfected polygonal-shaped HaCaT cells with a nucleus containing several nucleoli. Infected HaCaT cells in panel A2 appeared similar to uninfected cells at 2 hpi with no noticeable morphological differences. Strain L2 434 and US151 (L2) replicated faster serovar E, with approximately 26 matured RBs dividing by binary fission in the inclusion within 12 hpi (A3, B3, and C3). EBs appeared within 36 hpi (A5). EBs continued to accumulate within the inclusion at 48 hpi (A6). Similar ultrastructural events to L2 434 were observed in HaCaT cells infected US151 (L2) (Fig 3) from 2 to 24 hpi. However, there were numerous EBs at 36 and 48 hpi observed in HaCaT cells infected with L2 434 (panel A5 and A6) compared to US151 (L2) (B5 and B6) and serovar E (C5 and C6).

Discussion

The chlamydial developmental cycle has been well characterized microscopically. However, the signals that stimulate conversion from EB to RB and mechanisms associated with the regulation of intracellular development remain unclear [27]. It has been shown that the synthesis of numerous proteins occurs throughout the chlamydial developmental cycle, while other proteins are strictly associated with mid- and late-stage differentiation [10]. Early-, mid- and late-cycle genes represent a subset of genes that are important in understanding key events in the differentiation processes that control the developmental cycle [12]. Here, we used native host cell lines to investigate the *in vitro* chlamydial gene expression during the developmental cycle to understand the different pathogenicity of the LGV biovar of *C. trachomatis* and its G(genital) biovar in keratinocytes 37°C. This study used HaCaT cell line, ATCC reference strain (L2 434), and 2 clinical isolates (LGV L2 US151 and genital biovar E) isolated in a male patient presenting with genital ulcer and urethritis [23]. We observed different transcriptional expression of chlamydial genes post-infection with L2 434, US151, and serovar E. In addition, L2 434, US151, and serovar E strains presented the normal chlamydial replication cycle comprising EB and RB within 36 hpi.

For the first time, the *in vitro* expression of the *groEL-1, incB, pyk-F, hctA, tal* and *omcB* genes of *C. trachomatis* reference strain L2 434 and clinical isolates US151 (L2) and serovar E were studied using HaCaT cells. *groEL-1* expression levels remained fairly constant between 2 and 36 hpi for all tested strains. However, strain E showed increased *groEL-1* expression levels at 48 hpi. *groEL-1* is known to increase during nutrient deprivation which occurs early in the developmental cycle [13]. The increase observed in *groEL-1* at 48 hpi may be attributed to the loss of energy and nutrients during the differentiation of RBs to EBS late in the developmental cycle [13]. Cells infected with L2 434 had over a 30-fold increase in *incB* expression level at 2 hpi, compared to L2 US151 in which a constantly lower level of expression was observed. This was in agreement with the chlamydial growth rate observed using the TEM. Lack of induced expression of *incB* in our clinical LGV isolate may suggest that this isolate use one or more *inc* proteins other than *incB*. In addition, the difference between reference strain and clinical isolates may due to the modification of strain LGV 434 during numerous passages in cell culture. Furthermore, *incB* expression levels peaked at 12 hpi for strain E. The difference in the pattern
of gene expression observed for strain E could be correlated with the fact that the primary target for genital serovars are epithelial cells of the genital tract, and in this study, keratinocytes were used.
The expression of tal and pyk- F genes was observed at different levels amongst the tested chlamydial strains. pyk- F expression was most abundant at 2 hpi in all tested chlamydial strains, indicating that energy is required immediately following the invasion of chlamydia into the keratinocytes. In contrast, there was no expression of pyk- F in the early phase in HeLa cells infected with serovar E. The expression of tal was highest at 2 and 12 hpi in HaCaT cells with strain E, which correlated with rapid growth and division of RBs as observed in the ultra-structural analysis of the chlamydial developmental cycle. This was also consistent with the TEM observations. Our results suggest that tal and pyk- F genes were required to support energy metabolism and growth throughout the chlamydial developmental cycle in keratinocytes.

Two late-cycle genes that have been reported to be expressed from 24 hpi were also analyzed. These are, and omcB that encodes a cysteine-rich outer membrane protein that interacts with the MOMP to form this complex which involves extensive protein cross-linking through the formation of cysteine bonds [12]. We also observed high expression of hctA, a gene that encodes a chlamydial histone-like protein and mediates chromosomal condensation during the differentiation of RBs to EBs, at 2 hpi and decreased by 1- fold at 12 hpi for L2 434 and serovar E. High hctA expression observed in this study could be due to the highly condensed chlamydial chromosome in the early phase of the developmental cycle. Previously, it has been shown that the expressions of hctA gene was reduced as condensed chromatin of EB was dispersed during differentiation into pleomorphic RB [20]. Previous studies reported that hctA is not expressed until 24 hpi in HeLa cells [10, 11]. However, our study shows that hctA was expressed throughout the chlamydial developmental cycle but the level of expression increases from 24 hpi in keratinocytes. Another gene (omcB) expressed in the late-cycle was also observed at 2 hpi with L2 434. At 12 hpi the expression level was suppressed but upregulated again at 24 hpi. The expression of omcB gene is only found in EBs as the component of the disulfide-linked outer membrane protein complex that confers structural stability to EBs [10]. Thus, the early expression of omcB observed in this study suggests that chlamydia was still in an EB form at 2 hpi in keratinocytes. This was consistent with the TEM, which showed numerous EBs at 36 hpi.

Taken together, the observed differences in transcriptional expression of chlamydial genes between reference strain (L2 434) and clinical isolates (US151 and serovar E) and inconsistency with published data may not be attributed exclusively to the type of model or the MOI used, but to several other factors. These include carryover mRNA that may be present in EBs, high-passage numbers, different regulatory systems for gene expression in HaCaT cells compared to HeLa cell lines, and the origin (site of infection) of the isolated strain. Furthermore, in our population, LGV presents in the primary stage as a painful genital ulcer without the tendency to resolve spontaneously. This could mean that there is a genetic difference between LGV strains that is responsible for this difference in clinical presentation [5]. Lastly, the mRNA decay rate was not investigated in this study.

**Conclusion**

Our study showed different gene expression patterns between clinical isolates and reference strain during *in vitro* infection of the immortalized human keratinocyte, suggesting that keratinocytes are also appropriate to interrogate cell differentiation, growth kinetics, and gene expression of *C. trachomatis* infection. Extensive understanding of the chlamydia intracellular biology, including temporal expression patterns of genes during *in vitro* and *in vivo* infections is needed to develop novel therapeutic strategies against *C. trachomatis* infections and disease.
Supporting information

S1 Table. Processing schedule for TEM.
(DOCX)

S2 Table. Mean fold changes for six chlamydial genes in HaCaT cells.
(DOCX)

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