International Journal of Medical Microbiology 305 (2015) 378–382

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Short Communication

Expansion of the *Chlamydia trachomatis* inclusion does not require bacterial replication

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ARTICLE INFO

Article history:
Received 17 December 2014
Received in revised form 14 February 2015
Accepted 22 February 2015

Keywords:
Chlamydia trachomatis
Chemical biology
Inclusion expansion
Bacterial replication

ABSTRACT

Chlamydia trachomatis replication takes place inside of a host cell, exclusively within a vacuole known as the inclusion. During an infection, the inclusion expands to accommodate the increasing numbers of *C. trachomatis*. However, whether inclusion expansion requires bacterial replication and/or *de novo* protein synthesis has not been previously investigated in detail. Therefore, using a chemical biology approach, we herein investigated *C. trachomatis* inclusion expansion under varying conditions *in vitro*. Under normal cell culture conditions, inclusion expansion correlated with *C. trachomatis* replication. When bacterial replication was inhibited using KSK120, an inhibitor that targets *C. trachomatis* glucose metabolism, inclusions expanded even in the absence of bacterial replication. In contrast, when bacterial protein synthesis was inhibited using chloramphenicol, expansion of inclusions was blocked. Together, these data suggest that *de novo* protein synthesis is necessary, whereas bacterial replication is dispensable for *C. trachomatis* inclusion expansion.

Introduction

Chlamydia trachomatis is the causative agent of several sexually transmitted diseases (Haggerty et al., 2010) as well as the eye infection trachoma (Burton and Mabey, 2009). *Chlamydia* is an obligate intracellular bacterial pathogen that requires a eukaryotic host cell to subsequently grow and develop. Within a eukaryotic cell, *Chlamydia* resides in a membrane-bound vacuole known as the inclusion. Following invasion, *Chlamydia* alters the features of the internalized vacuole, which allows the bacteria to avoid degradation through the endocytic pathway (Fields and Hackstadt, 2002). Throughout the course of infection the inclusion expands as the number of chlamydial cells increase. The inclusion expansion has been suggested to be dependent on host cell lipid metabolism (Derre et al., 2011; Robertson et al., 2009), host cell cytoskeletal proteins, vimentin and actin (Kumar and Valdivia, 2008a), and secreted bacterial effector proteins (Jorgensen and Valdivia, 2008; Mital et al., 2013), which may manipulate processes in the host cytoplasm that are required for optimal inclusion expansion. It is possible that expansion of the chlamydial inclusion is linked to bacterial replication (Fields and Hackstadt, 2002; Kumar and Valdivia, 2008b), and in fact, inclusion area has been used to indirectly measure chlamydial replication and growth (Engström et al., 2013; Nguyen et al., 2011; Tietzel et al., 2009; Volceanov et al., 2014). However, a potential link between inclusion expansion and bacterial replication has not been proven experimentally or characterized under different growth conditions.

Acquisition of host cell metabolites is critical for chlamydial intracellular growth and development. Host cell glucose-6-phosphate (G-6P) appears to be important for *C. trachomatis* infectivity (Iliffe-Lee and McClarty, 2000; Omsland et al., 2012, 2014; Saka et al., 2011), and recently we identified an inhibitor, KSK120, which is a 2-pyridone inhibitor that blocks *C. trachomatis* infectivity by affecting its G-6P metabolism. Current data indicate that the G-6P transporter UhpC is targeted by KSK120 and that this compound may block import of G-6P into *C. trachomatis*. Moreover

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http://dx.doi.org/10.1016/j.ijmm.2015.02.007

and interestingly, we also observed that KSK120 might decrease bacterial replication without affecting the expansion of formed *C. trachomatis* inclusions (Engström et al., 2015). Thus, to potentially reveal novel insights on the regulation of inclusion expansion, we have further characterized the effect of compound KSK120 on *C. trachomatis* infections.

Material and methods

Cell culture, bacterial infections and compound KSK120

The HeLa cell line (DSMZ) was grown at 37 °C (5% CO_2) in RPMI 1640 (Sigma) supplemented with 10% FBS (Sigma) and 20 mM HEPES. *Chlamydia trachomatis* serovar LGV-L2 454/Bu (ATCC VR902B) and *C. trachomatis* serovar D were grown in HeLa cells and elementary bodies were purified as previously described by Caldwell et al. (1981) and stored in SPG buffer (0.25 M sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid). For infection experiments, HeLa cells were infected with *C. trachomatis* diluted in Hanks balance salt solution (HBSS) (GIBCO®, Invitrogen) with a multiplicity of infection (MOI) of 0.5 for 1 h at 37 °C (5% CO_2). Subsequently HBSS was removed and complete RPMI media supplemented with compound KSK120 or DMSO was added to the infected cells. Cycloheximide was excluded in all experiments. KSK120 was synthesized and handled as previously described (Engström et al., 2015). KSK120 was dissolved in dimethyl sulfoxide (DMSO, Sigma) to a final concentration of 20 mM, and stored at room temperature unexposed to light for a period of up to 2 months. Prior to the experiments, compounds were diluted in pre-warmed RPMI media to a concentration of 10 μM. Dilution of KSK120 in pre-warmed media is necessary to avoid compound aggregation. Chloramphenicol (Cml, Sigma) was dissolved in water to a final concentration of 20 mg/ml. At 24 hpi, media without Cml was removed from infected cells and subsequently replaced with fresh media containing 200 μg/ml Cml.

Determination of C. trachomatis *inclusion size and* C. trachomatis *replication*

At varying time points, postinfection, media was completely removed and infected cells were washed once in PBS before fixation in 100% methanol (room temperature) for 5 min. Background fluorescence signals were reduced by blocking the samples with 5% BSA for 1 h. Next, samples were incubated with primary anti-major outer membrane (MOMP) antibody (gift from Ken Fields) and primary anti-heat shock protein 60 (Hsp60) antibody (Santa Cruz Biotechnology). An LRSC-conjugated anti-rabbit antibody and a FITC-conjugated anti-mouse antibody (both from Jackson ImmunoResearch Laboratories) were used as secondary antibodies against MOMP and Hsp60, respectively. DNA was stained with 200 nM 4′,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted on glass slides with fluorescence mounting media (Dako). Images were obtained using confocal laser scanning microscopy using identical settings (Nikon Eclipse C1 plus). Identical contrast adjustments of images were carried out using Adobe Photoshop software (Adobe Systems Inc.). Inclusion areas were measured from acquired confocal transmission images that were processed using EZ-C1 software (Nikon), as previously described (Engström et al., 2013). Representative data are from one hundred *C. trachomatis* inclusions. *C. trachomatis* replication was determined by Real-Time quantitative PCR (qPCR) (iQ5 Real-Time PCR Detection System; Bio-Rad Laboratories), using SYBR-green chemistry (WWR). A total of 20 ng of DNA template were used in all qPCR reactions, and all samples were run in triplicate (Engström et al., 2010). Primers amplifying *omcB* were used to quantify *C. trachomatis* genomic DNA and primers amplifying *gapdh* were used to quantify host cell genomic DNA. Subsequently, *C. trachomatis* DNA was normalized against host cell DNA (Ouellette et al., 2006), and DMSO treated infections were arbitrarily set to 1. DNA was isolated from three independent experiments.

Results and discussion

C. trachomatis *replication and inclusion expansion are impaired to varying extents by compound KSK120*

We recently observed that KSK120-treatment altered the distribution of *C. trachomatis* serovar LGV-2 within the inclusion. The usual homogeneous distribution of *C. trachomatis* serovar LGV-2 becomes patchy, lacking uniformity (Fig. 1A). This observed effect was not accompanied by a change in the size or morphology of individual bacteria (Engström et al., 2015). However, it was unclear whether KSK120 affected inclusion size, bacterial replication or both. Therefore, in this study we quantified inclusion areas in the presence or absence of compound KSK120. We have included *C. trachomatis* serovar LGV-2 and serovar D in these experiments, which both cause urogenital infections. It is well known that *C. trachomatis* inclusions can fuse if two bacteria infect a single host cell. Therefore, we used a MOI of 0.5, to minimize the influence of inclusion fusion in our analysis. The compound was added immediately following HeLa cell infection and inclusion areas were measured at 48 and 60 hpi. Quantification of inclusion areas was possible through a combination of obtained transmission images that visualized the inclusion membrane and bacterial markers that labeled *C. trachomatis* (Fig. 1A). At 48 hpi, we observed a modest reduction in inclusion sizes of serovar D (15%) whereas the reduction was more pronounced in LGV-2 (28%). Because inclusions in non-treated infected cells began to lyse after 48 hpi, but inclusions in KSK120-treated infections remained intact, it was difficult to compare inclusion areas between KSK120-treated and non-treated infections at 60 hpi. Nonetheless, at 60 hpi, we observed that inclusion areas in KSK120-treated infections were significantly larger compared to KSK120-treated infections at 48 hpi (Fig. 1B). In addition, at 60 hpi, we observed that KSK120-treated infections had larger inclusions compared to DMSO-treated infections at 48 hpi. Together, these data suggest that KSK120-treatment slightly but significantly delays expansion of *C. trachomatis* inclusions.

To assess if KSK120 impaired bacterial replication we quantified genome equivalents at 48 hpi using qPCR and determined that KSK120 strongly inhibited replication of LGV-2 (10-fold reduction, Fig. 1C), and to a lesser extent serovar D (~3-fold reduction, Fig. 1C). Thus, we concluded that KSK120 affects *C. trachomatis* replication. Intriguingly, whereas KSK120 extensively reduced *C. trachomatis* replication it did not reduce the size of inclusion areas to the same extent (*i.e.* for LGV-2 at 48 hpi, genomic DNA was reduced by 90% while inclusion sizes were only reduced by 28%), indicating that bacterial replication is not a major determinant of inclusion expansion.

Inclusion expansion is dependent on protein synthesis but not C. trachomatis *replication*

Our previous data indicated that inclusion expansion did not necessarily depend on bacterial replication, however it was unclear if inclusions could expand in the absence of bacterial replication. To assess if inclusions could expand in the absence of *C. trachomatis* replication, we quantified genomic DNA and inclusion area sizes, in the presence or absence of KSK120 at different time points. Samples were collected at 24, 36 and 48 hpi. In control-treated infections an increase in bacterial replication correlated with larger