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Subtle changes in host cell density cause a serious error in monitoring of the intracellular growth of *Chlamydia trachomatis* in a low-oxygen environment: Proposal for a standardized culture method

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ABSTRACT

We monitored *Chlamydia trachomatis* growth in HeLa cells cultured with either DMEM or RPMI medium containing 10% FCS under 2% or 21% O_2 conditions for 2 days. Bacterial numbers, host cell numbers, and fibrosis-related gene expression in the host cells were estimated by an inclusion forming unit assay, a cell counting assay, and a PCR array, respectively. In contrast to RPMI, bacterial growth under low oxygen conditions in DMEM rapidly decreased with increasing host cell density. The addition of supplements (glucose, glutamine, vitamin B12, D-biotin, non-essential amino acids, glutathione) to the media had no effect. The growth of host cells in DMEM under low oxygen conditions rapidly decreased, although the cells remained healthy morphologically. Furthermore, the downregulation of 17 genes was observed under low oxygen in DMEM. Whereas no effect on bacterial growth was observed when culturing in RPMI medium at low oxygen, and the downregulation of three genes (*CTGF*, *SERPINE1*, *JUN*) was observed following bacterial infection compared with the uninfected control cells. Thus, our findings indicate the need for carefully selected culture conditions when performing experiments with *C. trachomatis* under low-oxygen environments, and RPMI (rather than DMEM) is recommended when a low host cell density is to be used, proposing the major modification of cell culturing method of *C. trachomatis* in a low-oxygen environment.

1. Introduction

Obligate intracellular bacterium *Chlamydia trachomatis* is the leading cause of bacterial sexually transmitted diseases worldwide, with an estimated 100 million chlamydial infections detected annually (Ziklo et al., 2016; Geneva: World Health Organization, 2011). Such infections are often asymptomatic in women (Imai et al., 2010; Haggerty et al., 2010; Qayum and Khalid-Bin-Saleem, 2013) and can therefore be left untreated, resulting in ascending infection with fibrosis, which is responsible for ductal obstruction, pelvic inflammatory disease, and infertility (Vodstrcil et al., 2015; Siracusano et al., 2014; Da Ros and Schmitt Cda, 2008). It is well known that a low-oxygen environment is required for the induction of fibrosis (Menon et al., 2015), although whether infection is involved in the induction remains unknown. However, accumulating evidence suggests that a low-oxygen environment plays a central role in stimulating host cell signaling pathways involved in metabolism and inflammation, which are responsible for initiating fibrosis via stabilizing hypoxia-inducible factor 1α (HIF-1α), an oxygen sensor in mammalian cells (Semenza, 2010; Rupp et al., 2007; Kim et al., 2006). It is therefore reasonable to suggest that the intracellular growth of *C. trachomatis* may modulate the signal pathway governed by HIF-1α in human fallopian tube cells, facilitating fibrosis.

C. trachomatis possesses a unique developmental cycle, consisting of the elementary (EB) and reticulate body (RB) forms, differentiated from EB to RB (or re-differentiated from RB to EB) in inclusion bodies surrounded by the membrane vesicle (Rockey and Matsumoto, 2000). After infection, the maturation process in infected host cells is known to

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; RPMI, Roswell Park Memorial Institute medium; IFUs, inclusion-forming units; Glu, glucose; Gln, glutamine; GG, glucose + glutamine; PCR, polymerase chain reaction; RT, reverse transcriptase

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require actin remodeling (Dunn and Valdivia, 2010; Jewett et al., 2006; Balañá et al., 2005), lipid metabolism (Stehr et al., 2013; Elwell and Engel, 2012; Saka and Valdivia, 2012), and inflammatory responses (Ziklo et al., 2016; Entrican et al., 2014; Cochrane et al., 2010). However, chlamydial studies in a low-oxygen environment are limited and difficulties are encountered in even assessing its intracellular growth. However, a few studies have shown that *C. trachomatis* adapts well to a low-oxygen environment, such as that found in the urogenital tract of women (Jerchel et al., 2014; Shima et al., 2011; Juul et al., 2007; Peters et al., 2005). Meanwhile, it is well recognized in low-oxygen environment that cellular metabolism such as glycolysis prompting ATP generation required for chlamydial growth can be up-regulated by stabilizing HIF-1α, which is a master transcriptional factor in the environment (Palmer and Clegg, 2014; Papandreou et al., 2006). Therefore, the cells left into culture under low-oxygen condition may rapidly consume substances in the cultures, inevitably responsible for deteriorating chlamydia growth into limited culture medium. Thus, accurate evaluation of chlamydial growth in a low-oxygen environment is a crucial issue for understanding chlamydial dynamics in the urogenital tract, although it has not been standardized among researchers.

In the present study, we carefully monitored *C. trachomatis* growth in immortal human epithelial HeLa cells and fibrosis-related host responses in a low-oxygen environment. We showed that in contrast to normal atmospheric conditions, subtle changes in host cell density can lead to serious errors in the monitoring of the intracellular growth of *C. trachomatis* in a low-oxygen environment, proposing the major modification of cell culturing method of *C. trachomatis* in a low-oxygen environment.

2. Materials and methods

The epithelial cell line HeLa was purchased from ATCC (Manassas, VA, USA). The cell line was maintained at 37 °C in 5% CO_2 in DMEM (Sigma) containing 10% heat-inactivated fetal calf serum (FCS), 10 μg/ml gentamycin, 10 μg/ml vancomycin, and 1 μg/ml amphotericin B (Sigma), according to a previously published protocol (Roblin et al., 1992). *C. trachomatis* D/UW3 Cx strain (VR-885) was purchased from ATCC, and propagated as described previously (Kubo et al., 2012). The number of infectious progeny for *C. trachomatis* was determined as inclusion forming units (IFU) by counting chlamydial inclusions formed in HeLa cells using a fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-*Chlamydia* antibody specific for *Chlamydia* lipopolysaccharide (with Evans Blue) (Denka Seiken) (Kubo et al., 2012).

HeLa cells were adjusted to a concentration of 2×10^5 cells/well and simultaneously infected with *C. trachomatis* at a multiplicity of infection (MOI) of 2 by centrifugation for 450 ×*g* at room temperature. After washing to remove non-infecting bacteria, the cells at a concentration of 1 to 20×10^4 cells/well were incubated with DMEM (D6046) or RPMI (R8758) containing 10% FCS, gentamycin (10 μg/ml), vancomycin (10 μg/ml), and amphotericin B (0.5 μg/ml) for 2 days at 37 °C under conditions of either 21% or 2% O_2. For some experiments, cells were cultured in DMEM plus supplements (vitamin B12, D-biotin, non-essential amino acids, glutamine, and glucose) (See Table S1) or cycloheximide (final concentration 2 μg/ml). Cells were harvested and viability was determined by a cell counting assay (see below) and EB numbers were determined by an IFU assay. A low-oxygen environment (2% O_2) was created using a dedicated camber MIC-101 (Billups-Rothenberg). The mixed gas containing 2% and 21% O_2 used as a control consisted of 2% O_2, 5% CO_2, and 93% N_2 or 21% O_2, 5% CO_2, and 74% N_2, respectively. In addition, pH values in culture mediums were also monitored in some experiments with a hand-type's pH meter, twinpH (Horiba Ltd.); the values was measured < 5 min after removed from the camber to minimize affect of atmosphere.

To determine the morphology of chlamydial inclusions, cells stained with specific antibodies were observed using a conventional [IX71 (Olympus)] or a confocal laser microscope [LSM510 (Carl Zeiss Japan Group)] (Kubo et al., 2012). Cell Counting Kit-8 (Dojindo) was used to determine cell viability according to the protocol described in the manufacturer's instruction. Briefly, water-soluble tetrazolium salt, WST-8, is reduced by dehydrogenase activity in cells to give a yellow colored formazan dye, which is soluble in tissue culture media. The intensity of the color is measured at $OD_{450\ nm}$ as an indicator of cell viability.

The Human Fibrosis RT^2 Profiler PCR Array kit equipped with 84 genes (Table S2) was used to determine the expression of fibrosis-related genes (Qiagen), according to the protocol described in the manufacturer's instructions. In brief, cells (20×10^4 cells/well in DMEM; 5×10^4 cells/well in RPMI) with or without *C. trachomatis* infection were incubated for 24 h under 2% or 21% O_2 conditions. After incubation, the cells were collected and then total RNA was extracted using the High Pure RNA Isolation Kit (Roche). An RT reaction was performed on 500 ng of RNA and the synthesized cDNA was analyzed by Applied Biosystems StepOne Plus real-time PCR system to determine the changes in fibrosis-related genes. The values were expressed as relative fold changes compared with the control array. According to the protocol, more than or less than two-fold changes compared with the control were defined as meaningful changes. Each of the values was expressed as the average of two independent experiments.

Comparison of the total IFU numbers between oxygen conditions and between experimental groups was conducted using a Bonferroni/Dunn test and Student's *t*-test, respectively. A *p*-value of < 0.01 was considered significant.

3. Results

First, by assessing bacterial numbers using an IFU assay and confocal fluorescence microscopy observations, we compared the growth of *C. trachomatis* (MOI 2) in HeLa cells (5×10^4 cells/well of a 24-well plate) cultured either in DMEM or RPMI medium in a 21% or 2% oxygen environment (Fig. 1A). The growth of *C. trachomatis* was gradually dampened in DMEM compared with RPMI medium, in particular in the 2% oxygen environment 48 h after infection (Fig. 1B). However, even when cultured in DMEM, there was no significant difference in inclusion size between the 21% and 2% oxygen environments (Fig. 1C). Furthermore, all of the cells appeared healthy morphologically, although the growth of HeLa cells was slowed under hypoxic conditions compared with conditions of 21% O_2 (Fig. S1). Thus, although bacterial growth initially appeared the same, there was a significant difference in terms of bacterial numbers, with numbers being decreased particularly in DMEM under low-oxygen conditions.

In contrast to RPMI medium, DMEM has a low glucose concentration and lacks certain components such as glutamine, non-essential amino acids, and some vitamins (Table S1). We therefore assessed whether supplementation of these components into DMEM could restore bacterial growth to the levels observed in RPMI medium under low oxygen (2% O_2) conditions (Fig. 2A). However, regardless of supplementation, bacterial growth did not recover to the levels observed under 21% O_2 conditions (Fig. 2B). Despite this, it was noted that in contrast to supplementation of DMEM with vitamin B12, D-biotin, and non-essential amino acids or glutamine alone, the addition of glucose, which is the most effective energy source, had an impact on bacterial growth (Fig. 2C). This result indicated that the lack of bacterial growth in DMEM under low oxygen was likely due to the rapid starvation of glucose arresting host cell growth, presumably in turn resulting in a decrease in bacterial intracellular growth because of the requirement for ATP energy from the host cells (Rockey and Matsumoto, 2000). This implied that changing the host cell density may have an impact on bacterial growth in HeLa cells in low oxygen environments.

To investigate the effect of host cell density changes, we compared the growth of *C. trachomatis* when cultured with host cells at a range of seeding concentrations (from 1×10^4 to 20×10^4 cells/well in a 24-