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Chlamydia Trachomatis Promotes 3T3 Cell Differentiation into Adipocytes

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. There is experimental and clinical evidence showing that some viral and bacterial pathogens are linked to the accumulation of excessive body fat and obesity.
Objectives. The aim of the study was to investigate the ability of *C. trachomatis* to propagate in the pre-adipocyte cell line and induce its differentiation into fat cells.
Material and Methods. 3T3 L1 pre-adipocytes or McCoy cells were plated and infected with *C. trachomatis*. The cell monolayers were further studied by immunofluorescent and quantitative RT-PCR methods.
Results. *C. trachomatis* can efficiently propagate in 3T3 L1 cells, a mouse pre-adipocyte cell line. The morphological characteristics of chlamydial growth revealed in 3T3 L1 cells with the monoclonal chlamydial MOMP-specific antibody resembled those seen in McCoy cells, a classic cell line used for chlamydial research. The number of chlamydial 16S rRNA copies detectable in the lysates of McCoy and 3T3 cells infected with *C. trachomatis* was almost identical, suggesting similar efficiency of pathogen propagation in both cell lines. Moreover, there was a significant increase in aP2 mRNA transcript levels as well as moderate induction of SCD-1 mRNA in the total RNA extracted from the infected 3T3 L1 cells 48 h following the pathogen inoculation. The increased expression of the adipogenic markers was also accompanied by lipid droplet accumulation in the *C. trachomatis* infected 3T3 L1 cells, suggesting their transformation into differentiated adipocytes.
Conclusions. The direct effect of the pathogen on fat cell progenitors observed in this work may explain abnormal fat deposition at the sites of chronic inflammation caused by *C. trachomatis* (**Adv Clin Exp Med 2014, 23, 4, 0–0**).

Key words: *C. trachomatis*, adipocytes, differentiation.

According to WHO statistics, excess weight and obesity have reached epidemic proportions, affecting at least 1.4 bn people worldwide. As a result approximately 2.8 m adults die each year due to obesity-related diseases. Although increased food intake and decreased level of physical activity are considered the most important primary causes of obesity, there is a growing body of evidence that some viral and bacterial pathogens may contribute to the pathogenesis of obesity. It has been shown recently that past adenovirus infection (Ad36) increases adiposity and affects glycemic control in humans [1]. Multiple experimental studies show that adenoviral infections – in particular those caused by the Ad-36 adenovirus – induce changes in insulin sensitivity, glucose uptake and expression of diabetes-related genes. Moreover, there is a traceable epidemiological link between some adenoviral infections and obesity [2, 3]. Among other viruses associated with a risk of obesity are canine distemper virus, Rous-associated virus 7, scrapie, Borna disease virus and SMAM-1 [4]. Some bacterial pathogens are also supposedly related to the development of obesity [5]. It has been reported recently that an endotoxin-producing isolate of *Enterobacter* derived from the gut of obese humans causes obesity when inoculated into germ-free mice [6]. Beside the systemic changes, infectious

agents may cause abnormalities in regional body fat portioning, resulting in lipodystrophy in cases of HIV infection [7]. Although inflammatory mediators seem to play a major role in the association between infections and adiposity [6], some viral pathogens can directly promote the differentiation of fat cells. In particular, Ad-36 has been shown to accelerate pre-adipocyte transformation into mature fat cells under *in vitro* conditions [8].

The present paper reports that *Chlamydia trachomatis,* an intracellular bacterial pathogen, can induce differentiation of pre-adipocyte cells into adipocytes.

Material and Methods

Reagents, Cell Lines and Pathogen

All reagents were purchased from Sigma-Aldrich unless otherwise specified.

McCoy and Hep2 cells were obtained from the European Collection of Cell Cultures (Salisbury, UK) and grown in 5% CO_2 in DMEM supplemented with 2 mM glutamine and 5% FCS. 3T3 L1 cells, a mouse embryonic cell line, were purchased from the HPA Culture Collection (catalog number 86052701, United Kingdom) and were grown in DMEM supplemented with 2 mM glutamine and 10% calf serum in 5% CO_2 at 37°C until confluence was reached, according to the supplier's instructions. To stimulate differentiation into adipocytes, additions of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM dexamethasone and 1μg/mL insulin were made to the confluent cultures. Strain L2/Bu434 of *C. trachomatis* was kindly provided by Dr. P. Saikku (University of Oulu, Finland). The pathogen was initially propagated in Hep2 cells and purified by Renografin gradient centrifugation as described by Galdwell et al. [9]. Chlamydial titers were determined by infecting Hep2 cells with 10-fold dilutions of thawed stock suspension. Purified elementary bodies (EB) of known titer were suspended in sucrose-phosphate-glutamic acid buffer [9] and used as inoculums for 3T3 or McCoy cells. 3T3 or McCoy cells were infected by inoculating the cell monolayers using stock solution of *C.trachomatis* at 1 MOI, with further centrifugation of the infected cells at 1500 rpm for 60 min. Cell monolayers were refed every 48 h. Infected cells were visualized after permeabilization by direct immunofluorescence using FITC-conjugated monoclonal antibody against major outer membrane protein (MOMP) (NearMedic Plus, RF). Inclusion-containing cells were visualized using a Nikon Eclipse 50i fluorescence microscope at ×1350 magnification.

Assessment of Bacterial Growth

In order to evaluate *C. trachomatis* growth in 3T3 or McCoy cells, the infected cell monolayers were grown in DMEM supplemented with 2mM glutamine and 10% calf serum in 5% CO_2 at 37°C and harvested after 24, 48, 72 and 144 h of the post-infection period. The infected cells were visualized with *C. trachomatis* MOMP-specific monoclonal antibody. RNA was extracted and analyzed as described below. Lipid inclusions were visualized on day 7 of the post-infection period with Sigma Sudan Black B Staining System.

RNA Extraction and Reverse Transcription

RNA was isolated from cell monolayers grown on 6-well plates using TRIZol (Invitrogen). Total mRNA pretreated with DNase I (DNA-free™, Ambion) and quantified on a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Wilmington, USA) was transformed into cDNA using random hexamer primers and a SuperScript III First-Strand Synthesis Kit (Invitrogen, Karlsruhe, Germany).

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

The 16S rRNA was studied as a constitutive marker of the chlamydial developmental cycle to compare the efficiency of *C. trachomatis* propagation in host cells using quantitative RT-PCR on an ANK 32 thermocycler (Syntol, RF). Primers for *C. trachomatis* 16S rRNA (sense-5'-GGCGTATTTGGGCATCCGAGTAACG-3', antisense-5'-TCAAATCCAGCGGGTATTAACCGCCT-3') were verified and used under thermal cycling conditions: 95°C for 10 min and 50 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 20 s. Serial dilutions of *C. trachomatis c*DNA extracted from chlamydia-infected Hep-2 cells were used as a standard for quantification. Among the mouse-specific genes studied were major genes related to lipid metabolism and adipocyte differentiation: aP2 (forward primer 5'-TGATGCCTTTGTGGGAACCT-3', reverse primer 5'-GCAAAGCCCACTCCCACTT-3'), PPARγ (forward primer 5'-ATCATCTACACGATGCTGGCC-3', reverse primer 5'-CTCCCTGGTCATGAATCCTTG-3') and SCD-1 (forward primer 5'-CATCATTCTCATGGTCCTGCT-3', reverse primer 5'-CCCAGTCGTACACGTCATTTT-3'). The results of the PCR analysis for the mouse-specific genes were normalized to mRNA values for mouse GAPDH

(forward primer 5'-AACTTTGGCATTGTG-GAAGG-3', reverse primer 5'-TGTGAGGGA-GATGCTCAGTG-3'). The mRNA expression levels in the host cells were referenced to the CT values in uninfected control cells grown under the same conditions. This reference value was taken as 1.00. Each cDNA sample was tested by PCR at least three times. All experiments were repeated at least twice. Representative sets of results are shown below.

Results

As can be seen from the results of the immunofluorescence analysis (Fig. 1), inoculation of *C. trachomatis* into 3T3 L1 and McCoy cell cultures led to active infection in the cell monolayers. In general, the morphology and dynamics of the *C. trachomatis* infection cycle were very similar in both cell lines and involved approximately 25–30% of the cells in the cell monolayers. In both cell lines, inclusion bodies started to appear after 24 h of the post-infection period, predominantly in the peripheral zone of the cytoplasm. Inclusion bodies were much larger and more densely stained 48 h after *C. trachomatis* inoculation and were accompanied by nuclear dislocation, while some immunopositive granules were observed outside of the cells. The most intense staining of intracellular inclusion bodies was seen after 72 h of the post-infection period, when most of the infected cells were round-shaped and also had multiple immunopositive extracellular granules surrounding

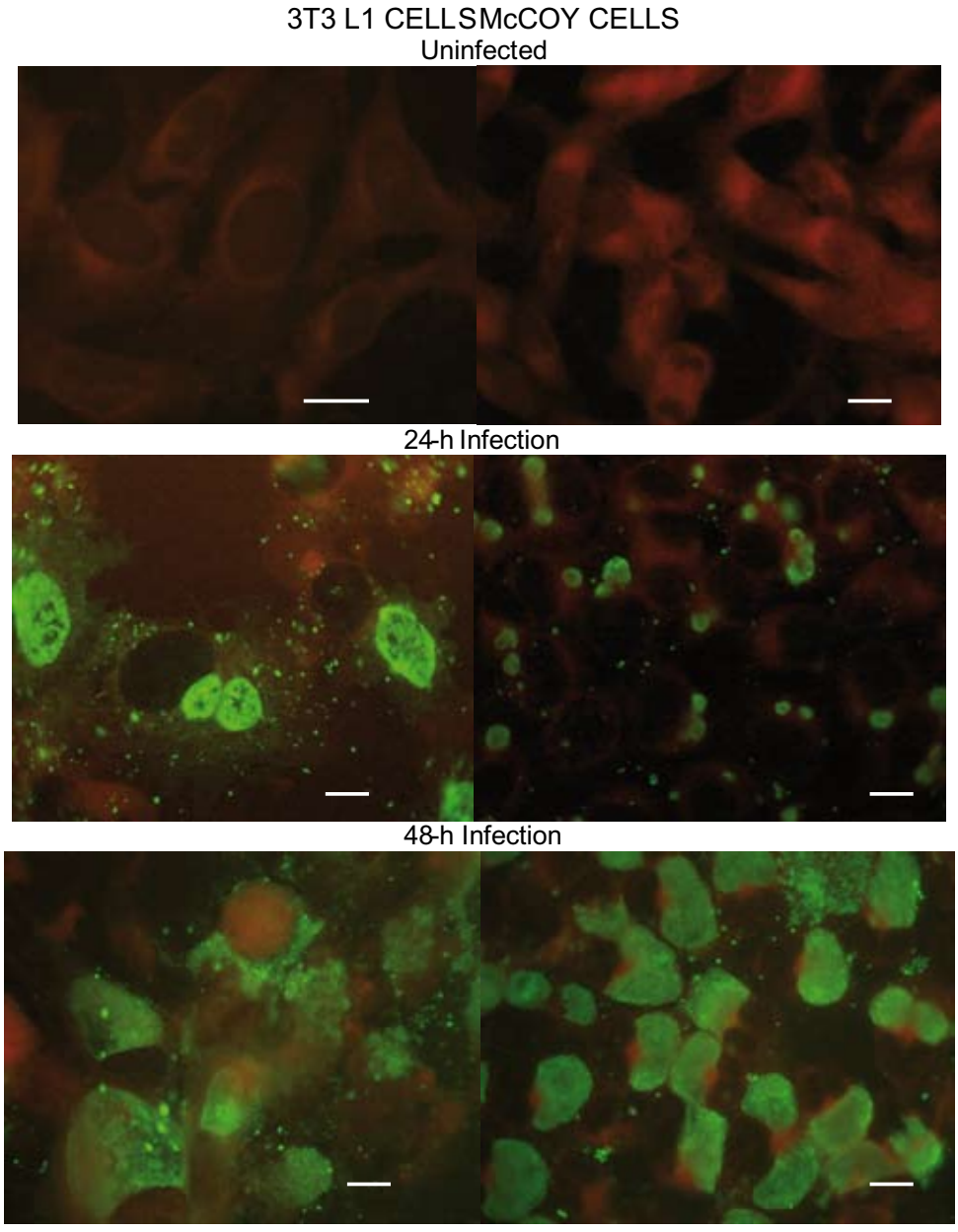

Fig. 1. Propagation of *C. trachomatis* in 3T3 L1 and McCoy cells

3T3 L1 and McCoy cells were set up, grown and processed for immunostaining with MOMP-specific monoclonal antibodies at the 0-, 24- and 48-h points of the post-infection period, as described in Materials and Methods

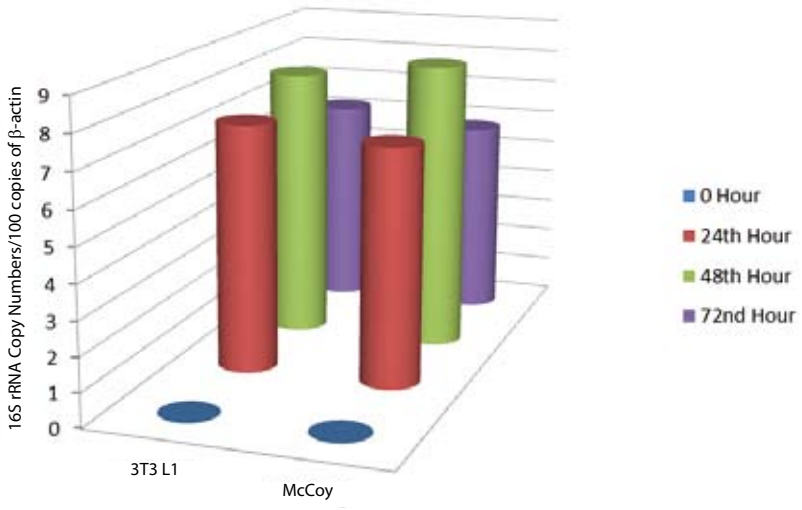

Fig. 2. 16S *rRNA* in 3T3 L1 and McCoy cells infected with *C. trachomatis*

3T3 L1 and McCoy cells were set up, grown and harvested at the 0-, 24-, 48- and 72-h points of the post-infection period. 16S rRNA was measured as described in Materials and Methods

them. Later points in time in the post-infection period were characterized by cytolytic changes in the infected cells, leading to an accumulation of cell debris with densely stained granules and further spread of the infection to newly infected adjacent cells (results not shown).

A nucleic acid amplification protocol was used in an attempt to quantify the intensity of chlamydial growth in 3T3 L1 and McCoy cells. As can be seen from Fig. 2, the number of chlamydial 16S rRNA copies detectable in the lysates of McCoy and 3T3 cells infected with *C. trachomatis* was almost identical throughout the whole post-infection period. This implies that the efficiency of propagation in the two host cell lines was equal.

In order to evaluate the effect of *C. trachomatis* on 3T3 cell differentiation, the mRNA adipocyte markers were studied in the total mRNA specimens extracted 48 h after inoculating *C. trachomatis* into the 3T3 L1 cell monolayers. As can be seen from Table 1, there was a significant increase in aP2 mRNA transcript levels, as well as moderate induction of SCD-1 mRNA. PPARγ mRNA was almost unchanged at the time point of the experiment shown.

To evaluate changes in lipid content, infected 3T3 L1 cells were double stained with Sudan black and further treated with MOMP-specific monoclonal antibodies on day 7 of the post-infection period. As Fig. 3 shows, there was neither visible lipid nor immunofluorescent staining in the uninfected 3T3 L1 cells. However, as can be seen from the lower panel of Fig. 3, 3T3 L1 cells infected with *C. trachomatis* had multiple lipid droplets surrounding the chlamydial inclusion body. A similar aP2 mRNA response and lipid droplet formation (on day 2 and day 7, respectively) was observed in uninfected 3T3 cells induced with insulin (results not shown).

Discussion

C. trachomatis infection is the most common sexually transmitted communicable disease in North America and Europe [10] with urogenital, pelvic and ocular manifestations, leading to infertility and preventable blindness if untreated [11]. However, most cases of *C. trachomatis* infections are asymptomatic, which explains exponential growth of affected individuals around the world. The overall median rate of *C. trachomatis* positivity worldwide is approximately 4.7%, with variations from 1.3% to 18.1% in different countries [12]. The existing limitations in treating *C. trachomatis* infections arise from antibiotic resistance and insufficient knowledge of the molecular mechanisms underlying the pathogenesis of this disease and its various complications.

Table 1. Fold changes in mRNA expression levels in 3T3 L1 cells infected with *C. trachomatis*

Experimental Condition	GAPDH mRNA	aP2 mRNA	SCD-1 mRNA	PPARγ mRNA
Control: uninfected 3T3 L1 cells	1	1	1	1
Infected 3T3 L1 cells	1	11.4	2.9	1.32

3T3 L1 cells were set up, infected with *C. trachomatis* and grown as described in Material and Methods. The cells were harvested at the 0- and 48-h points of the post-infection period for total RNA extraction. Mouse specific mRNAs were measured and normalized to GAPDH as described in Material and Methods.

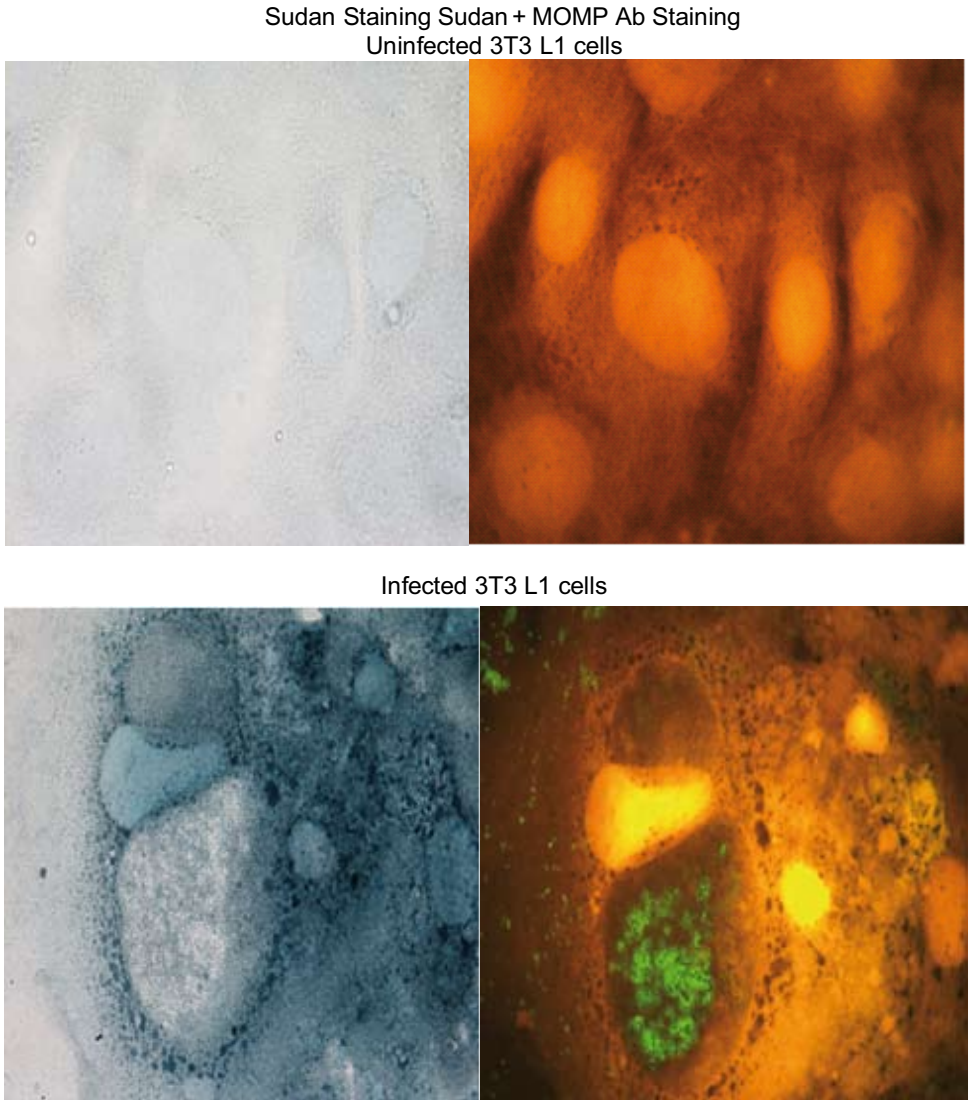

Fig. 3. Lipid droplet accumulation in 3T3 L1 cells infected with *C.trachomatis*

3T3 L1 cells were set up, grown and subjected to a double staining protocol (immunostaining with MOMP-specific monoclonal antibody and/or sudan staining) on day 7 after infection with *C. trachomatis*. Immunofluorescent images were obtained as described in Materials and Methods

In the present paper it is shown that *C. trachomatis*, a bacterial pathogen responsible for most cases of preventable blindness and urogenital infections in the world, can efficiently propagate and fully accomplish its infectious cycle in a pre-adipocyte cell line: 3T3 cells. This statement is solidly supported by the fact that the number of 16S rRNA copies detectable during the post-infection period in 3T3 cells is almost equal to that measurable in McCoy cells, a classic cell line used for chlamydial research for many decades.

However, the most important finding made in the current study is the fact that propagation of *C. trachomatis* in 3T3 cells promotes the differentiation of pre-adipocytes into fat cells. This conclusion is well supported by the results of the histochemistry analysis, which reveal the appearance of lipid droplets in the cytoplasm of 3T3 cells infected with the chlamydial pathogen, as well as the mRNA changes found in the infected 3T3 cells. According to the findings of the current study, infecting the 3T3 L1 cells with *C. trachomatis* led to an increase in aP2 transcript numbers with corresponding upregulation in SCD-1 mRNA which are well known molecular markers of adipocyte differentiation. These clear signs of 3T3 cell differentiation induced by *C. trachomatis* came as a big surprise in the current study, as recently published experiments with *C. pneumoniae* and 3T3 cells yielded negative results. According to Shi et al. [13], *C. pneumoniae* infection in 3T3 cells does not lead to the transformation of pre-adipocytes into fat cells under similar conditions to those used in the present study. Therefore, the current results illustrating the ability of *C. trachomatis* to induce adipocyte differentiation represent a novel finding, revealing a striking difference in the outcome of infections caused by two closely related but distinct chlamydial pathogens.

Although the results of the present study do not reveal the molecular mechanism underlying *C. trachomatis*-induced pre-adipocyte differentiation, there is a body of both experimental and clinical evidence supporting this observation and explaining it to some extent. In particular, it is widely believed

[14] that *C. trachomatis* relies heavily on the lipid metabolism of host cells and is capable of redirecting lipid trafficking and biosynthesis in mammalian cells [15]. *C. trachomatis* inclusions, even in host cells with limited ability to synthesize lipids, such as HeLa cells, have been shown to be surrounded by host lipid droplets with neutral lipids and tubular structures expressing adipocyte-differentiation-related protein, perilipin, Rab18 and other potential inducers of adipogenesis [16]. Moreover, from a clinical point of view, chronic inflammation caused by *C. trachomatis* infection is often accompanied by extensive remodelling of urogenital tissues and accumulation of cervicodorsal fat [17, 18]. Chronic pelvic inflammation is reportedly often associated with fat tissue deposits [19]. In particular, besides lymph node enlargement, an extensive mesenteric fat accumulation has been reported in patients with Fitz-Hugh-Curtis syndrome, a rare complication of *C. trachomatis* infection [20, 21].

Although at this stage it not clear what contribution *C. trachomatis* infection may make in the pathogenesis of systemic obesity, the direct effect of the pathogen on fat cell progenitors observed in this work may explain abnormal fat deposition at the sites of chronic inflammation caused by *C. trachomatis*. Further research is needed to clarify the possible role of *C. trachomatis* in body fat content regulation.

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