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Apoptotic effect on HeLa Cells produced by *Chlamydia trachomatis*-LPS

Efecto Apoptótico en Células HeLa Producido por el Lipopolisacárido (LPS) de Chlamydia trachomatis

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Abstract

The interaction between the lipopolysaccharide (LPS) of *Chlamydia trachomatis* and mammalian cells is still largely unknown. *Chlamydia trachomatis* is an obligate intracellular bacterium responsible for several diseases in humans and animals. In this work, thanks to the isolation of the lipopolysaccharide from two serovars of *Chlamydia trachomatis* (LGV1-LGV2) and using a nuclear supravital fluorescent stain (Hoechst 33258), it was possible to investigate the apoptotic effect on HeLa cells. This work shows the apoptotic effect on HeLa cells when they were exposed to *C. trachomatis*-LPS from two serovars at concentrations equal to or higher than 0.5 µg/mL for a period of 48h. and also the lack of cellular response in the absence of *C. trachomatis*-LPS or in the presence of LPS obtained from other bacteria. Additionally, the use in equal conditions of polymyxin B, known as an inhibitor of bacterial LPS, showed a decrease of the apoptotic effect in such cells indicating that the cellular response observed was produced by *C. trachomatis*-LPS. These results support the theory that the LPS from *C. trachomatis* could be responsible for the toxic effect on cervical cells infected by these bacteria.

Key words: *Chlamydia trachomatis, LPS, apoptosis, nuclear supravital fluorescent stain.*

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Resumen

La interacción entre el lipopolisacárido (LPS) de *Chlamydia trachomatis* y las células de mamíferos permanece sin ser dilucidado. *Chlamydia trachomatis* es una bacteria intracelular responsable de diversas enfermedades en los humanos y animales. En este trabajo mediante el aislamiento del lipopolisacárido de dos serovares de *Chlamydia trachomatis* (LGV1-LGV2) y usando una coloración Supravital fluorescente (Hoechst 33258) fue posible investigar la respuesta de las células HeLa. El efecto apoptótico que sufren este tipo de células fue visible cuando fueron expuestas a dicho LPS en concentraciones iguales o mayores que 0,5 µg/mL por un periodo de 48 horas, sin embargo se observó la falta de repuesta celular en su ausencia o en presencia de LPS de otras bacterias. Adicionalmente, el uso en iguales condiciones de polimyxina B conocido como un neutralizador de la acción del LPS demostró una disminución del efecto apoptótico en dichas células, indicando que la respuesta celular observada fue producida por *C.trachomatis*-LPS. Los resultados de este trabajo le dan fuerza a la teoría de que el LPS de *C. trachomatis* pudiera ser el responsable del efecto tóxico que se observa sobre las células cervicales infectadas con esta bacteria intracelular.

Palabras clave: *Chlamydia trachomatis, LPS, Apoptosis, Coloración nuclear supravital fluorescente.*

Introduction

Apoptosis or programmed cell death is a process in which cells play an active role in their own death and in attaining and maintaining a normal organism

(1). It is an innate, controlled and regulated mechanism of eukaryotic cell which play a major role in many physiological and pathological processes in response to a variety of stimuli (2, 3).

After receiving specific signals instructing the cells to undergo apoptosis a number of distinctive biochemical and morphological changes occur in the cell. Thus, these biochemical changes are reflected in morphological changes in the cell and are marked by a series of reorganization characteristics such as chromatin condensation, loss of cell volume and membrane blebbing which are some of the most evident morphological changes of apoptotic cells (1.3).

Chlamydiae are obligate intracellular bacteria with a cell wall with an inner and outer membrane similar to any of Gram Negative bacteria and responsible for various acute and chronic diseases in animals and humans. Ocular infection and sexually transmitted diseases in humans resulting in cervicitis, salpingitis, pelvic inflammatory disease, postpartum endometritis, male urethritis non-gonococcal, and epididymitis are commonly caused by *Chlamydia trachomatis*. In almost all Gram negative bacteria cell wall, there is an amphiphilic material known as lipopolysaccharide (LPS), which is one of the major components of the outer membrane and is implicated in the inflammatory response through a potent activation of macrophages (4). Chlamydial-LPS is similar to the rough forms of enterobacterial LPS which consists of the lipid A and core regions and appears to be responsible for the generation of cytokines in which *C. trachomatis* interacts with tissue phagocytes during the initiation of the infection process (5). The endotoxic activity of LPS depends mainly on the structure of the lipid A moiety (6), however Chlamydial lipid A has less endotoxic potency than enterobacterial lipid A, which was

attributed to the lower number of acyl chains and to their unusual, long-chain of fatty acids (5, 7). This reason could also explain the asymptomatic course of *C. trachomatis* infected patients. In addition, there is evidence that LPS extracted from *C. trachomatis* (serovar LGV) has a great influence on sperm motility and viability during *in vitro* conditions (8, 9).

In spite of the great progress made on the chemical structure on Chlamydia-LPS (10, 11) little is known of its endotoxic effect and its role in chlamydial infection. The aim of the present work was to investigate the presence of apoptotic effect on HeLa cells produced by *C. trachomatis*-LPS using a nuclear supravital fluorescent stain (Hoechst 33258) which enters the cell nucleus and binds DNA only when the plasma membrane is damaged. Hoechst 33258 is a benzimidazole compound and a part of a family of fluorescent stains for labelling DNA in fluorescence microscopy, this dye is water soluble and is utilized to document apoptosis by visualizing chromatin condensation and nuclear shrinkage due to a change in membrane permeability and form a complex with DNA but not RNA due to its specifically binding to the A-T base pairs in ds DNA resulting in increase in fluorescence emission. Hoechst 33258 has been used previously as an early detection of *C. trachomatis* growing in tissue culture (12) and also as an important tool in apoptosis detection (13, 14).

Material and Methods

Cell Lines: McCoy cells (Mouse fibroblast cell line) and HeLa 229 cells (Human Cervical Carcinoma Cell Line) were obtained from the American Type Culture Collection (ATCC) (Manassas, Va.) and maintained according to the supplier's instructions.

Cell Culture: McCoy cells were cultured in 75-cm^2 flasks and HeLa cells were cultured in trak bottles with LPS-free coverslips in Eagle minimal essential medium (EMEM) with L-glutamine (Bio-Whittaker 12-611F) supplemented with 10% v/v heat inactivated fetal calf serum (FCS) (Life Technologies) and maintained at 37°C in an atmosphere of 5%CO_2. *C. trachomatis* serovar LGV-L1 and LGV-L2 were propagated in a semiconfluent McCoy cell sheet for 48 - 72 h in EMEM media with 10% v/v FCS and cyclohexamide [2ug mL^{-1}]. EB's were harvested and purified as previously described (15).

LPS production: LPS from *C. trachomatis* serovars LGV-L1 and LGV-L2 were extracted according to a method previously used (16). In order to investigate the successful extraction of LPS, 20 µL was run on a 14% polyacrylamide gel at 150 V and commercial LPS from *Escherichia coli* 055 (Sigma) was used as a control. After running the gel, it was fixed in 40% methanol plus 10% acetic acid overnight followed by washing 5-6 times (for 5 min) in the fixative solution containing 0.7% periodic acid (BDH Chemicals). The gel was then stained using the silver staining Bio Rad Kit. A Limulus Amebocyte Lysate kit (LAL) (Bio-Whittaker) was used to quantify the LPS. The method was performed as recommended by the manufacturer. The concentrations of LPS were calculated according to the standard curve and its final concentration was recorded as µg/mL.

LPS Cell Toxicity Assay: HeLa cells grown on coverslips as described previously, were tested using three concentrations (0.1, 0.5, 1.0 µg/mL) of the extracted LPS from serovars LGV-L1 and LGV-L2, the commercial *Pseudomona aeruginosa*-LPS (Sigma), and *E.coli* 055-LPS (Sigma). The LPS was diluted to the final concentration in EMEM medium with 10% FCS and exposed to the cells which

were incubated at different intervals of time, (3, 6, 24, and 48 h.) under the normal conditions described earlier. Staurosporine 1 µM (Sigma) was used to induce apoptosis in HeLa cells and was used as a positive control. The apoptotic inducer was in contact with the cells the same length of time as LPS; cells with only EMEM medium supplemented with 10% FCS were used as a negative control. After incubation period, the coverslips were then stained with Hoechst 33258.

Apoptosis Assessment: the level of apoptosis was determined by measuring the number of cells showing apoptotic features such as chromosomal condensation and fragmentation after staining with Hoechst 33258 (Sigma) using a fluorescence microscope. The cells after being exposed to LPS or staurosporine for the stated period of time were fixed with 2% paraformaldehyde in PBS for 30 min, followed by permeabilization with 0.5% saponin in PBS for 30 min and then stained with Hoechst 33258, (2 µM) prepared in EMEM medium without FCS for 30 min. The cells were washed three times with PBS, and mounted onto slides using Vectashield mounting medium for fluorescence (Vector Lab,Inc.Burlingame, CA). This procedure was performed at room temperature. The percentage of apoptotic cells was calculated as the number of apoptotic cells compared to the number of total cells counted. Images from Hoechst stained samples were acquired under a 100X objective lens using a Leica™ Fluorescence microscope equipped with a Leica™ camera.

Statistical analysis: The data shown are means ± standard errors of triplicate cultures. The statistical significance of the differences between each dose and its negative control was examined by a one-way analysis of variance (ANOVA) on transformed data.

Results

In the present work was possible to obtain good quantities of *C. trachomatis*-LPS and according to the LPS quantification tests performed, the concentration of *C. trachomatis*-LPS from serovars LGV-L1 was 50 µg/mL and for serovar LGV-L2 was 500 µg/mL.

The apoptotic effect caused by *C. trachomatis*-LPS on HeLa cells was assessed by examining the chromosomal condensation and fragmentation of the cells with a fluorescence microscope using Hoechst staining (Figure 1). No significant effect was observed when the cells were treated for a period of 3 or 6 h, neither at concentrations of LPS lower than 1.0 µg/mL, nor with any of the commercial LPS used. After a 24 h incubation period, tested cells exposed to *C. trachomatis*-LPS exhibit a similar response to those cells exposed to the commercial *P.aeruginosa*-LPS and *E.coli*-LPS which showed a low level of apoptotic cells throughout experiments (<3%). However after 48 h a significant difference was recorded for the *C. trachomatis*-LPS treated cells and the level of apoptotic cells for serovars LGV-L1 and LGV-L2 at concentrations of 0.5 µg/mL (6% and 16%) and at 1µg/mL (27% and 32%) respectively were observed. Also, the level of apoptosis cells observed with LGV-L2 is significantly higher than the apoptosis showed for the cells treated with LGV-L1 and that was visible with all the concentrations tested. Staurosporine treated cells used as a positive control showed a high level of apoptosis (11% and 44%) at 24 and 48h of exposure respectively and the untreated cells showed a low level of apoptotic cells (less than2%) even at 48h (Figure 2).

HeLa cells were also tested with the highest concentration of *C. trachomatis*-LPS in the presence of polymyxin B for a

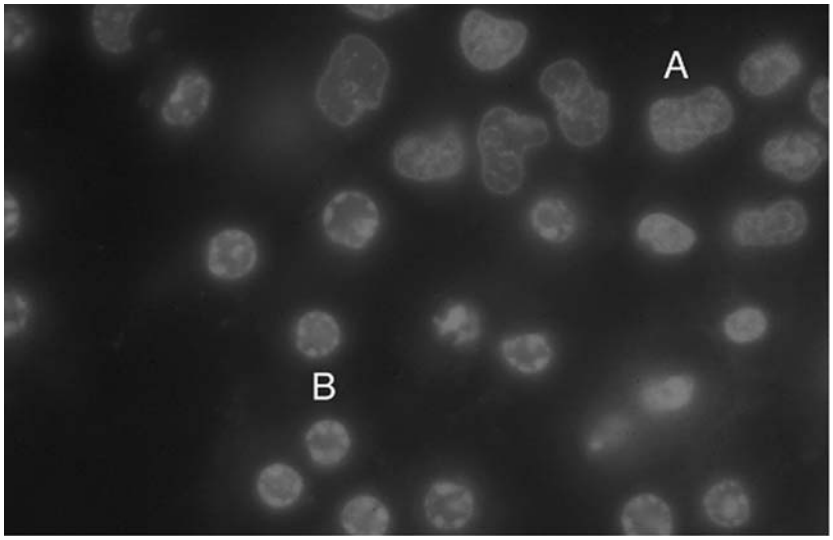

Figure 1. Fluorescence micrographs of HeLa cells treated with *C. trachomatis*–LPS using a Leica™ camera mounted on a Leica™ fluorescence microscope. (A) Normal HeLa cells (B) HeLa cells showing apoptotic feature using Hoechst staining.

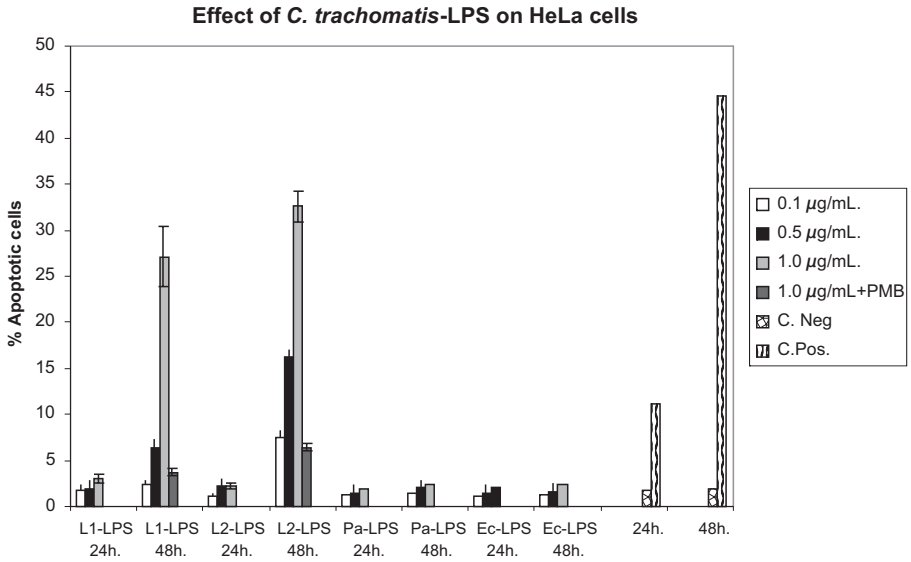

Figure 2. Apoptotic index of HeLa cells in presence of LPS from 2 serovars of *C.trachomatis* (LGV-L1 and LGV-L2) and two commercial LPS at different periods of time when stained by Hoechst 33258. (Pa-LPS: *Pseudomona aeruginosa* commercial LPS, Ec-LPS: *Escherichia coli* commercial LPS, PMB : Polymyxin B.)

period of 48h. and the apoptotic effect showed by the cells (3% for LGV-L1 and 6% for LGV-L2) decreased significantly in relation to the cells treated only with *C. trachomatis*-LPS (Figure 2).

Discussion

Lipopolysaccharide (LPS) is the major virulence factor of Gram-negative bacteria. Induction of apoptosis of mammalian host cells has been shown in several bacteria, in-

cluding *E.coli* (17), *Shigella flexneri* (18), *Staphylococcus aureus* (19) and *Helicobacter pylori* (20). However, the role of *C. trachomatis*-LPS in the cell death process remains unclear. Reports of the interaction between *C. trachomatis* and mammalian host cells have been made but the effects of LPS on apoptotic cell death differ among cell types and cell lines and to the best of our knowledge the apoptotic effect of *C. trachomatis*-LPS on epithelial cells has not been studied before.

The apoptotic effect during Chlamydial infection of epithelial cells has been observed previously. *C. psittaci* has been found to induce apoptosis in infected epithelial cells and macrophages in vitro (21, 22). *C. trachomatis* has been reported to induce apoptosis *in vitro* in LLC-MK2 cells (21) and *in vivo* during genital tract infection in mice (23). Schoier et al. (2001) (24), shows that *C. trachomatis* induces apoptosis in infected McCoy and HeLa cells and also that the apoptosis that occurred in uninfected cells was induced late in the *Chlamydial* developmental cycle, beyond 24h post-infection. Interestingly, those works show that at 48 h the induction of apoptosis by infected cells became noticeable and was significantly higher than after 24 h which also correlates with our findings.

It is well known the difficulty to obtain massive quantities of *C. trachomatis*-LPS due to the nature of the bacteria; however in this work a reasonable amount from both serovars were produced and the apoptotic effect of Chlamydial-LPS on HeLa cells, a cell line derived from epithelial cells was demonstrated, and apoptotic features were visible when the cells were in contact with *C. trachomatis*-LPS from either of the serovars LGV-L1 or LGV-L2 at concentration of 0.5 µg/mL or higher for a period of 48h. Our findings also show that HeLa cells were not sensitive to *E.coli*-LPS and *P. aeruginosa*-LPS at concentrations of 1.0 µg/mL at that time.

These observations could suggest that the apoptosis on the cells is induced by LPS and that the potency of the *C. trachomatis*-LPS is considerable higher in the cervix cells than the apoptosis produced by LPS from other kind of bacteria. The use of polymyxin B (PmB), a cationic, cyclic peptide antibiotic, which inhibits biological activities of bacterial LPS through high affinity binding to the lipid A moiety (25, 26) was used to test its inhibitory effect on Chlamydial-LPS and the reduction of apoptotic cells was statistically significant indicating that the effects observed were primarily as a result of LPS activity.

This finding highly correlates with recent studies of the significance of *Chlamydia*-LPS in human sperm and how it affects sperm viability (27, 8, 9) and offer support to further LPS studies and its action on affected cells. However, the complex reactions between host cells and *Chlamydia sp*. has increased the need for better understanding in this area and despite several studies supporting both anti and pro apoptotic activities described for different serovars and cell lines this matter still unclear and is currently under research by different groups.

In summary, the effect of LPS differs among cell type or cell line and this study demonstrates that *C. trachomatis*-LPS from serovars LGV-L1 and LGV-L2 is responsible for inducing apoptosis in a dose and time dependant manner in HeLa cells. Also, the results here presented offer support to the theory that the LPS from *C. trachomatis* could be responsible for the toxic effect on cervical cells infected by these bacteria.

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