Synthesis of von Willebrand Factor Antigen in Chlamydia pneumoniae-Infected Endothelial Cells

Chaicharoen Tantanate, M.D.*, Wanida Wongtiraporn, M.D.*, Nipattra Tragonlugsana, B.Sc.**, Narumol Chuensomboon, M.T.*, Sontana Siritantikorn, Dr.rer.nat**
*Department of Clinical Pathology, **Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

Correspondence to: Sontana Siritantikorn
E-mail: sissr@mahidol.ac.th

ABSTRACT

Objective: The main objective of our study was to evaluate the response of endothelial cells infected with Chlamydia pneumoniae (C. pneumoniae), by using von Willebrand factor (vWF) antigen as a marker for endothelial damage and dysfunction. Another objective was to evaluate the effect of cycloheximide on C. pneumoniae infectivity and vWF secretion from human umbilical vein endothelial cells (HUVECs).

Methods: HUVECs were harvested. After first passage, the HUVECs were inoculated with C. pneumoniae in three concentrations of cycloheximide (0, 1, and 2 μg/mL). At 24, 48, and 72 hours post-inoculation, supernatants from each HUVEC culture well were collected and measured for vWF antigen by sandwich ELISA as well as non-infected HUVECs were used as controls. C. pneumoniae infectivity was evaluated by indirect immunofluorescence technique and polymerase chain reaction.

Results: The cycloheximide-treated HUVECs resulted in greater infection compared to the non-treated HUVECs. Means of vWF antigens from HUVECs infected with C. pneumoniae were not different from those of non-infected HUVECs. However, there was a significant change in vWF secretion when different concentrations of cycloheximide were used in the culture system.

Conclusion: From our study, the results of vWF antigen secreted from HUVECs infected with C. pneumoniae were not different from those of non-infected HUVECs. However, there was a significant change in vWF secretion when different concentrations of cycloheximide were used in the culture system. C. pneumoniae infectivity was evaluated by indirect immunofluorescence technique and polymerase chain reaction.

Keywords: Atherosclerosis; Chlamydophila (or Chlamydia) pneumoniae; cycloheximide; human umbilical vein endothelial cell; von Willebrand factor

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Atherosclerosis is an inflammatory disease of large and medium-sized arteries that causes much morbidity and mortality. The pathogenesis of this condition has been explained by many mechanisms.1,2 The main mechanisms are the abnormalities in lipid metabolism, endothelial dysfunction, inflammatory and immunologic factors, plaque rupture, and smoking and in recent years, inflammation caused by infection have been proposed. Many organisms are involved in atheroma formation including Chlamydia pneumoniae (C. pneumoniae, previously called Chlamydia pneumoniae), cytomegalovirus, and Helicobacter pylori. Some studies add hepatitis A virus and herpes simplex virus type 1 and type 2 as contributors of atherosclerosis.3,4,5 Concerning C. pneumoniae, there are many evidences on its relationship to atherosclerosis. Firstly, a study by Saikku et al. in 1988 demonstrated that patients who had coronary artery diseases (CAD) were more likely to have a significant level of anti-C. pneumoniae antibodies than those who did not have a CAD condition.6 Following this study, several papers have been published to demonstrate the association of C. pneumoniae and atherosclerosis and its consequences. Apart from seroepidemiological study, other evidences are used to support the link between C. pneumoniae and atherosclerosis such as in vivo animal model studies and direct detection of C. pneumoniae within atherosclerotic lesions by immunohistochemistry, electron microscopy, in situ hybridization or amplification of bacterial DNA by polymerase chain reaction (PCR).6,7
There are many possible mechanisms used to explain the pathogenesis of atherosclerosis caused by *C. pneumoniae*. One of these is the endothelial dysfunction. It is found that infection of this obligate intracellular gram negative bacterium can cause exposure of bacterial endotoxin or lipopolysaccharide (LPS) and heat shock protein 60 to endothelial cell and macrophage which contributes to the stimulation of many inflammatory cytokines that initiate atheroma formation. Following this event, the injured endothelium will produce many adhesion molecules such as E-selectin or endothelial-leukocyte adhesion molecule 1 (ELAM-1), intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) as well as procoagulant-like tissue factor. von Willebrand factor (vWF) is an adhesive glycoprotein stored in specific secretory organelles known as Weibel-Palade bodies that undergo regulated exocytosis in response to inflammation and vascular injury. It has been estimated that only 5% of newly synthesized vWF is stored in cultured HUVECs but secreted continuously. Previous data demonstrated that plasma vWF level increment is correlated with endothelial dysfunction or damage in atherosclerosis and coronary artery disease. Some *in vitro* studies showed that the inflammatory response of endothelial cells from various stimulations can aggravate the production of vWF. The effect of *C. pneumoniae* infection and vWF stimulation due to endothelial dysfunction were studied. Cook et al. investigated the association between *C. pneumoniae* infection and cardiovascular disease as well as various markers including vWF. They demonstrated the correlation of *C. pneumoniae* IgG level and severe hypertension, but not the plasma vWF antigen. Gabriel et al. also showed that *C. pneumoniae* infection did not relate to the change of plasma vWF and platelet activity, although other inflammatory markers increased. This result was in consistent with the study by Rothenbacher et al. in that various infections were not associated with vWF activity. However, there is no in vitro study about the correlation between vWF secreted from HUVECs and *C. pneumoniae* infection. The sensitive cell lines for cultivation *C. pneumoniae* are cycloheximide-treated HEp-2 cells, NCI-H 292, and HL cell lines. Other cell lines such as McCoy, HeLa 229 and BHK-21 are also commonly used. Although HUVECs are not the cell line of choice for *C. pneumoniae* culture, it was shown that this organism can infect HUVECs.

**MATERIALS AND METHODS**

**Human umbilical vein endothelial cell (HUVEC) culture**

HUVECs were collected from human umbilical veins according to the Jaffe’s method. Human umbilical cord was cut off at the suture marks at both ends and then the umbilical vein was cannulated with the catheters at both sides by aseptic technique. After removing the retained blood clots by perfusing the vein with PBS, the vein was filled with 0.05% trypsin-EDTA and incubated at 37°C for 20 minutes. The released cells were collected in 50-ml tube and centrifuged at 1,000 rpm for 10 minutes. The cell pellet were suspended in M-199 media (Gibco, USA) supplemented with 20% fetal bovine serum (FBS, Gibco, USA) and cultured in 35 mm tissue culture Petri dishes at 37°C in a 5% CO₂ humidified atmosphere.

**Chlamydia pneumoniae organisms**

*C. pneumoniae* from stock cultures were inoculated and propagated in a HEp-2 cell monolayer that was cultured in Earle’s minimal essential medium (EMEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), streptomycin 50 μg/mL and cycloheximide 2 μg/mL at 37°C in 5% CO₂ humidified atmosphere before being inoculated into the endothelial cells. The quantification of organisms was performed by indirect immunofluorescent staining and reported as inclusion forming unit per ml (IFU/mL). In this study, the *C. pneumoniae* quantities of 2.2 x 10⁴ IFU/mL were used.

**Chlamydia pneumoniae infection of human umbilical endothelial cells**

Upon the confluence of HUVECs, these cells were then trypsinized into a 24-well plate at a concentration of 400,000 to 500,000 cells per well and maintained for 24 to 48 hours in M-199 with 20% FBS. Before being infected with *C. pneumoniae*, the endothelial culture media in each well was removed. The 2.2 x 10⁴ IFU/mL of *C. pneumoniae* were inoculated into the endothelial cells. Following centrifugation at 800 g and incubation at 37°C for 1 hour, the inocula were removed. Infected HUVECs were washed twice with phosphate buffered saline (Gibco, USA) and cultured in M-199 media containing 10% inactivated FBS and cycloheximide at the concentration of 0.1 and 2 μg/mL. After inoculation, the infected HUVECs were incubated at 37°C in a 5% CO₂ humidified atmosphere for 72 hours. The control, non-infected HUVECs were incubated in the same concentrations and conditions of cycloheximide like those of infected cells. During the incubation, 250 μL of supernatant from each well was collected at the time of inoculation, 24, 48 and 72 hours post-incubation for the detection of von Willebrand factor. After 72 hours, the trypsinized cells from each well were quantitated to determine any change of cell volume by counting on counting chamber.

Infection of *C. pneumoniae* in HUVEC cells at 72 hours post infection (hpi) was confirmed by indirect immunofluorescent staining and nested polymerase chain reaction (PCR). Indirect immunofluorescent staining was performed by trypsinization of HUVECs from each well and stained with monoclonal antibody against *C. pneumoniae* (DAKO, Denmark). The target gene for nested PCR is 16s rRNA gene of *C. pneumoniae*. It was performed by using outer primers: C07 (5’ to 3’: ACG GAA TAA TGA CTT CGG) and C08 (5’ to 3’: TAC CTG GTA CGC TCA ATT). Inner primers were C11 (5’ to 3’: ATG ATG ACT TCG GTT GTT ATT) and D01 (5’ to 3’: CGT CAT CGC CTT GGT GGG CTT). The final amplified products sized 221 base pairs. The PCR programmed was heating at 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 55°C for 30 seconds, and 72°C for 1 min.

**Detection of von Willebrand factor antigen**

vWF antigen from each well at different time (24, 48, and 72 hpi) was measured by a sandwich enzyme-linked
immunosorbent assay (ELISA). In short, the 100 μL of supernatant from each well collected at different times was added into the prepared well of the microtiter plate which was coated with rabbit anti-human vWF in duplicate. Then the plate was incubated at 37°C for 150 minutes and washed for removal of excess antigens. After completing the incubation, 100 μL of anti-human vWF IgG conjugated with peroxidase was added into each well and incubated for 150 minutes at 37°C. Then the plate was washed and 100 μL of substrate solution was added to each well. The reactions were read by an ELISA reader at λ of 492 nm and reported as OD. The recorded values were calculated into percentage of standard by using a standard curve.

RESULTS

Infection of C. pneumoniae on HUVECs

Micrographs of HUVECs before infection and after inoculation with C. pneumoniae were recorded under inverted and fluorescent microscopes, respectively. Before inoculation, HUVECs were arranged as monolayers in polygonal morphology with close contact borders in a cobblestone arrangement (Fig 1). After 72 hpi, HUVECs were stained with fluorescent monoclonal antibody to C. pneumoniae. Positive cells were presented as single, round and apple-green color in the cytoplasm. Control HUVECs showed no positive infected cell (Fig 2A). Less number and less fluorescent intensity of positive cells were found in the absent of cycloheximide culture (Fig 2B) compared to infected cells in cycloheximide cultures at the concentration of 1 μg/mL (Fig 2C) and 2 μg/mL (Fig 2D). Slightly differences of the number of cells at pre-inoculation and 72 hpi were observed (data not shown).

The infection of HUVECs by C. pneumoniae at 72 hpi was then confirmed by using nested PCR. After amplification, HUVECs that were infected with C. pneumoniae and gave the DNA products as shown in Fig 3. Non-infected HUVECs showed no product on amplification.

Quantities of von Willebrand factor antigen from HUVECs in various conditions

The data of vWF antigen level was presented in the form of the mean and % coefficient of variation (%CV) from the duplicated experiments. The amounts of vWF antigen production in various conditions at different times after C. pneumoniae inoculation are summarized in Fig 4.

Fig 1. HUVEC monolayer examined under inverted microscope before infected with C. pneumoniae. These cells are in large, flat, polygonal-shaped cells with a cobblestone arrangement.

Fig 2. HUVEC culture after 72 hpi examined under fluorescence microscope. No apple green, fluorescent color of HUVECs was observed in control HUVECs (A), C. pneumoniae-infected HUVECs in culture without cycloheximide showed weak intensity and less numbers of positive cells (B) compared to infected HUVECs culture in the presence of 1 μg/mL of cycloheximide (C) and 2 μg/mL of cycloheximide (D) which showed strongly intensity and high number of fluorescent positive cells.

Fig 3. Nested PCR of HUVECs at 72 hpi. Lane N and P represented negative and positive control for 16s rRNA gene of C. pneumoniae respectively. Lane A, HUVECs that were not infected with C. pneumoniae showed no amplification product. In lane B, C, and D, HUVECs that were infected with C. pneumoniae but varied in cycloheximide concentration (0, 1, and 2 μg/mL respectively) showed positive products at the same level.

In this study, the amounts of vWF antigen were continuously increased according to the time after the inoculation. The initial amount of vWF antigen level in each condition was approximately 40 % according to the quantity of vWF antigen found in fetal bovine serum in culture media. At 24 hpi, the levels of vWF in all conditions did not diverge as much as at 72 hpi. The levels of vWF production are higher in the culture system without cycloheximide than in that with cycloheximide. At 72 hpi, the amounts of vWF of HUVECs without cycloheximide were approximately twice of those produced by HUVECs that had cycloheximide in the culture system. The difference in cycloheximide concentration (1 and 2 μg/mL) did not
seem to significantly affect the production of vWF antigen. Moreover, the presence of *C. pneumoniae* infection in HUVECs did not affect the production of vWF antigen.

**DISCUSSION**

Improve the ability of *C. pneumoniae* to infect cell culture in many studies is cycloheximide. It is widely used in many researches as a glutarimide antibiotic which inhibits DNA and protein synthesis in eukaryotic cells but does not affect prokaryotic cells. It is suggested that in cultures treated with cycloheximide, the number of chlamydial inclusions is generally higher than those treated by other methods. In our study, HUVECs which were treated with cycloheximide before inoculation with *C. pneumoniae* gave a stronger intensity of fluorescent staining than the cells that did not use cycloheximide. This confirms that the infectivity of *C. pneumoniae* in condition where cycloheximide is present is better than those without it. We also found that different concentrations of cycloheximide at 1 and 2 μg/mL did not show a more distinctly staining intensity or level of vWF antigen. This demonstrated the inhibitory effect of cycloheximide on vWF synthesis. A previous study by Paleolog *et al.* also showed that pre-incubation of IL-1 and TNF-α-treated HUVECs with 5 μg/mL of cycloheximide abolished the enhancement of vWF secretion. However, Schorer *et al.* found that cycloheximide had no effect on vWF secretion of IL-1 and LPS-treated HUVECs. Tsai *et al.* investigated two forms of vWF from HUVECs and found that cycloheximide inhibits the release of low molecular weight vWF but not the high molecular weight vWF. In our study, so far, we found that cycloheximide was useful for *C. pneumoniae* inoculation to HUVECs, but an appropriate dose of cycloheximide was needed to validate the analysis about protein synthesis in the culture system with cycloheximide. From these results, we suggested that the infection of HUVECs by *C. pneumoniae* did not affect endothelial damage as demonstrated by vWF secretion compared to non-infected HUVECs. The limitation of this study was the non-variety doses of *C. pneumoniae* and other surrogate markers of endothelial damage and dysfunction which contributed to atherosclerosis were not investigated in this study. Therefore further studies on atherogenic pathogenesis of endothelial damage by *C. pneumoniae* are still needed for the benefit of patient's care and monitoring.
REFERENCES

การสร้างข้อมูลในบริเวณแพทเทอร์นแอคทิฟโดยช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์
ชัยชัยวิประเทศพงษ์ ชัยชัยวิประเทศพงษ์, เป็นนิสิตศิลปศาสตร์ มหาวิทยาลัยนิวโมนิทิช์ Dr.ร.น.ร.ร.น.ร.ร.น.ร.

วัตถุประสงค์: เพื่อศึกษาการครอบคลุมของช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ โดยใช้การวิเคราะห์แบบแพทเทอร์นแอคทิฟเป็นตัวแทนของการเกิดการทำงานของช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์

วิธีการ: ทำการเก็บช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ ที่มีช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ โดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ ที่มีช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ โดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ ที่มีช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ ที่มีช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ ที่มีช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ ที่มีช่องลมโดยใช้เกี่ยวกับกล้ามเนื้ออ่อนล้าน นิวโมนิทิช์ ที่มีช่องล